10/627,160 103

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 31/34, 31/44, 31/155, 37/26, 37/54, C07H 17/00, C12N 5/00, 9/02, C12Q 1/26, G01N 33/564

A1

(11) International Publication Number: WO 95/18609

(43) International Publication Date: 13 July 1995 (13.07.95)

(21) International Application Number: PCT/US94/00089

(22) International Filing Date: 4 January 1994 (04.01.94)

(60) Parent Application or Grant
(63) Related by Continuation
US 07/936,660 (CIP)
Filed on 28 August 1992 (28.08.92)

(71) Applicant *(for all designated States except US)*: CITY OF HOPE [US/US]; 1500 East Duarte Road, Duarte, CA 91010-0269 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NADLER, Jerry, L. [US/US]; 2445 Upper Terrace Road, La Crescenta, CA 91214 (US). NATARAJAN, Rama, Devi [IN/US]; 16837 East Dawn Haven Road, Hacienta Heights, CA 91745 (US).

(74) Agent: IRONS, Edward, S.; East Tower, Suite 701, 555 Thirteenth Street, N.W., Washington, DC 20004 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: HUMAN LEUKOCYTE 12-LIPOXYGENASE, MEDIATION OF ITS PATHWAY, AND CONSEQUENCES THEREOF

(57) Abstract

The discovery and regulation of a 12-LO isoform (hl 12-LO) expressed in human tissues and of a similar isoform in PVSMCs is described. Various ribozyme and chemical inhibitors of the hl 12-LO pathway are described. The involvement of this 12-LO pathway in AII induced hypertrophy is demonstrated. It is shown that HG culture potentiates the stimulatory effects of AII and HETE in vascular smooth muscles. A specific aspect of the invention is the discovery that activation of a 12-LO pathway in vascular smooth muscle accentuates AII induced vascular hypertrophic response in diabetes mellitus. Human therapy involving mediation of the hl 12-LO pathway is described.

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

HUMAN LEUKOCYTE 12-LIPOXYGENASE MEDIATION OF ITS PATHWAY, AND CONSEQUENCES THEREOF

This application is a continuation-in-part of application Serial No. 07/936,660 filed 28 August 1992.

This invention was made with government support under Grant No. DK 39721 RO1 awarded by the National Institutes of Health. The government has certain rights in the invention.

TABLE OF CONTENTS

D. Inhibition of 12-LO
   1. Chemical Inhibition of hl 12-LO
   2. Ribozyme Inhibition of 12-LO

E. Therapeutic Uses/Implications of Pharmacologic or Molecular Inhibition of Human Leukocyte 12-Lipoxygenase
   1. Inhibition of Angiotensin II-induced vascular, renal, cardiac and adrenal actions.
   2. Reduced Atherosclerosis (Primary) or Neointimal Proliferation Associated with Angioplasty or Cardiac Transplantation.
   3. Inhibition of Vascular and Kidney Disease Associated With Diabetes.
   4. Prevention of Pancreatic Islet cell Damage in Autoimmune (Type 1) Diabetes or during Islet Cell Transplantation to Reverse Diabetes.
   5. Prevention of Cancer Cell Growth and/or Metastases.
   6. Diagnostic Assays Utilizing hl 12-LO Immunogen.
   7. PDGF Enhancement of 12-LO Activity in VSMC.

I. FIELD OF INVENTION

This invention relates to a newly discovered form of 12-lipoxygenase RNA and protein in human adrenal, vascular smooth muscle cells, endothelial cells, monocytes, and pancreas, to the mediation of that enzyme and to therapeutic consequences of such mediation.

II. ABBREVIATIONS

LO = Lipoxygenase
12-LO = 12-Lipoxygenase

hl 12-LO	= Human Leukocyte 12-Lipoxygenase
hl 15-LO	= Human Leukocyte 15-Lipoxygenase
pl 12-LO	= Human platelet 12-Lipoxygenase
15-LO	= 15-Lipoxygenase
HETE	= Hydroxyeicosatetraenoic Acid
12 HETE	= 12-Hydroxyeicosatetraenoic Acid
12-HPETE	= 12-Hydroperoxyeicosatetraenoic Acid
HODE	= Hydroxyoctadecadienoic acid
GF	= Growth Factor
PDGF	= Platelet Derived Growth Factor
TGFB	= Transforming Growth Factor Beta
EGF	= Epidermal Growth Factor
HPLC	= High Pressure Liquid Chromatography
TNF	= Tumor Necrosis Factor 1
IL-1	= Interleukin-1
AII	= Angiotensin II
SMC	= Smooth Muscle Cells
VSMC	= Vascular Smooth Muscle Cells
PVSMC	= Porcine Vascular Smooth Muscle Cells
PKC	= Protein Kinase C
LDL	= Low Density Lipoprotein
mmLDL	= Minimally Modified Low Density Lipoprotein
RT-PCR	= Reverse Transcriptase Polymerase Chain Reaction
NG	= Normal Glucose
HG	= High Glucose
FN	= Fibronectin
GAPDH	= Glyceraldehyde-3-phosphate dehydrogenase

III. BACKGROUND

Recent studies in several animal species indicate that at least two forms of 12-LO exist (1,2). One form was originally isolated from human (3) and

bovine (4) platelets has to date been found exclusively in that cell type. The other known form of 12-LO was isolated from porcine leukocytes (5), porcine pituitary (6) and from bovine trachea.

IV. SUMMARY OF THE INVENTION

This invention includes the discovery and regulation of a 12-LO isoform (hl 12-LO) expressed in human tissues and of a similar isoform in PVSMCs. Various ribozyme and chemical inhibitors of the hl 12-LO pathway are described. The involvement of this 12-LO pathway in AII induced hypertrophy is demonstrated. It is shown that HG culture potentiates the stimulatory effects of AII and HETE in vascular smooth muscles.

A specific aspect of the invention is the discovery that activation of a 12-LO pathway in vascular smooth muscle accentuates AII induced vascular hypertrophic response in diabetes mellitus.

Human therapy involving mediation of the hl 12-LO pathway is described.

V. DETAILED DESCRIPTION OF THE INVENTION

A. Identification of hl 12-LO

The precise form of 12-LO expressed in human adrenal glomerulosa, pancreatic islets and other human tissues was identified utilizing a combination of immunologic and molecular approaches.

1. Description of Figures 1 to 7

Figure 1 depicts specific expression and regulation of porcine leukocyte-type 12-LO protein (72 kD) expression in normal human adrenal glomerulosa cells demonstrated by immunoblotting. Cells in 6-well dishes were serum-depleted for 24

hours by placing in DME/F12 + 0.2% BSA + 0.4% FCS without antibiotics. This medium was then again freshly added prior to the experiment followed by addition of AII. Incubations were run for 50 hours at the end of which cells were washed twice with ice-cold PBS and harvested by scraping. Adrenal glomerulosa cell pellets as well as HEL cell pellets (negative controls) were lysed and cytosol fractions (25 μg) electrophoresed along with authentic porcine leukocyte 12-LO (1 μg) and then subjected to western blotting using a specific antibody to a synthetic porcine leukocyte 12-LO peptide. Lane 1: Molecular weight markers. Lane 2: Authentic partially purified 12-LO. Lane 3: HEL cells. Lane 4: Untreated human adrenal glomerulosa cells. Lane 5: Human adrenal glomerulosa cells treated for 50 hours with AII 10^{-7}M. AII caused a 2.5 fold increase in 12-LO protein expression as determined by video densitimetry. Results shown are representative of four separate experiments showing 2-4 fold upregulation of 12-LO by AII using either antibody to the porcine leukocyte 12-LO-protein or to the synthetic 12-LO peptide.

Figure 2 depicts an autoradiogram of Northern blot analysis of RNA samples. Total RNA 20 μg (lanes 1 and 2) from normal adrenal cells were electrophoresed, blotted and hybridized with [^{32}P]-labeled porcine leukocyte 12-LO oligonucleotide probe. Molecular weight markers are 28S, 18S rRNA and 0.24-9.5 kb RNA ladder as indicated.

Figure 3 depicts in-situ hybridization of human leukocyte type 12-lipoxygenase mRNA in adrenal cortex. Figure 3A, positive staining with the anti-sense riboprobe, reveals predominant message in the glomerulosa of the adrenal cortex. Figure 3B,

negative control using the sense riboprobe showing no hybridization (magnification 200 X).

Figure 4 depicts comparison autoradiograms of PCR of cDNA for human 15-LO and cDNA for porcine leukocyte 12-LO. cDNAs samples were amplified for 25 cycles with specific primers for the gene (Table 1) and were hybridized with a labeled porcine leukocyte 12-LO oligonucleotide probe (panel A) or with a labeled human 15-LO oligonucleotide probe (panel B). Lane 1 is porcine leukocyte 12-LO primers on cDNA for porcine leukocyte 12-LO. Lane 2 is human 15-LO primers on cDNA for human 15-LO.

Figure 5 shows identification of leukocyte type 12-LO in human adrenal glomerulosa and mononuclear type cells (U937) using RT-PCR. RNA samples were amplified for 30 cycles with porcine leukocyte 12-LO primers. Membranes were hybridized with internal porcine leukocyte 12-LO oligonucleotide probe. Panel A, lane 1 represents total RNA from normal human adrenal glomerulosa using RT-PCR. Lane 2 is a negative control without template and Lane 3 is a negative control using human 15-LO cDNA. Samples in lane B are mRNA or total RNA from human U937 cells. Lanes 1 and 5 represent negative controls without reverse transcriptase (RT) for mRNA and total RNA respectively. Lane 2, mRNA and lane 6, total RNA are true RT-PCR. Lane 3 is a positive control using the porcine leukocyte 12-LO cDNA. Lane 4 is another negative control without RNA template.

Figure 6 shows RT-PCR analysis of leukocyte type 12-LO and platelet type 12-LO in HEL cells, U937-cells, IM-9 cells and glomerulosa cells (GC). Panel A and B are ethidium bromide-stained agarose gels, Panel C and D are autoradiograms of membrane hybridized with 12-LO oligonucleotide probe after

RT-PCR. Panels on the left are analysis of the leukocyte type 12-LO. All samples were amplified with β_2-microglobulin primers plus porcine leukocyte 12-LO primers except U937 RNA Samples were amplified with porcine leukocyte 12-LO primers alone. 404 bp and 333 bp represent amplified products of β_2 microglobulin and leukocyte 12-LO respectively. Lane 1 is size standards of a Hae 111 digest of ϕX174-pUC19. Lane 2 is a negative control without template. Samples in lanes 5, 9 and 10 are without RTase. The right panels are analysis of platelet type 12-LO. RNA samples were amplified with platelet 12-LO primers and GAPDH primers. 284 bp and 159 bp represent amplified products of GAPDH and platelet 12-LO respectively. Lane 6 is a negative control without RNA. Lane 7 is a size standard.

Figure 7 depicts regulation of 12-LO mRNA levels in human adrenal cells by AII determined by RT-PCR.

Figure 8 depicts regulation of 12-LO protein expression by AII in human aortic vascular smooth muscle (HVSMC).

Figure 9 illustrates the presence of leukocyte type 12-LO in human aortic smooth muscle and mononuclear cells and also shows induction of 12-LO expression by AII in human vascular smooth muscle cells (HSMC).

Figure 10 shows the release of 12-LO product 12-HETE by AII in human vascular smooth muscle cells.

Figure 11 depicts RT-PCR Southern blot analysis showing the presence of human leukocyte type 12-LO in human aortic endothelial cells. Lane 1, cDNA positive control. Lane 2, 12-LO expressed DNAase treated showing band is not from DNA contamination and Lane 3 is total RNA from endothelial cells showing 333 base pair product.

Figure 12 depicts RT-PCR, Southern blot analysis showing that human aortic endothelial cells do not express the 15-LO RNA but only the 12-LO RNA of leukocyte type. Presence of positive control amplification of 15-LO cDNA but complete absence of 15-LO RNA in two separate samples of human aortic endothelial cells is depicted.

2. Experimental Procedures

Culture and Incubation of Human Adrenal Glomerulosa Cells

Fresh normal human adrenal tissue samples were obtained from surgeries involving nephrectomies of renal carcinoma or adrenalectomies during retroperitoneal lymph node dissections. Tissue was obtained with IRB approval. The outer adrenal cortical tissue was separated by a pathologist with microscopic assistance under sterile conditions. The outer glomerulosa tissue was minced in DME/F12 medium containing 0.2% BSA, penicillin G (100 U/ml) and streptomycin (100 μg/ml). Cell suspensions were prepared as described earlier (7). The zona fasciculata contamination of these cultures was assessed to be minimal as judged by the very low levels of cortisol produced (8) as well as the very low levels of the 17 α-hydroxylase gene which is not active in the zona glomerulosa (9). Cells were plated on 6-well culture plates in DME/F12 containing 10% fetal calf serum (2 X 10^5/ml) and medium changed every 48 hours. Approximately 24 hours prior to an experiment, the medium was replaced with antibiotic-free DME/F12 containing 0.5% fetal calf serum (FCS) and 0.2% BSA.

Immediately prior to the incubations, the above low serum medium was freshly added. In some studies angiotensin II (10^{-7}M) (synthetic, human, Peninsula

Laboratories) was added and compared with control incubations. Incubations were carried out for 24 hours to examine LO mRNA expression and for 30 hours to examine LO protein expression. The cell monolayers were washed twice with ice cold phosphate buffered saline (PBS) and then processed for Western Analysis or RNA extraction as described below.

Culture of U937, IM-9 and HEL Cells

A monocyte-like human histiocytic lymphoma cell line, U937, was obtained from American Type culture Collection (ATCC CRL 1593). The human lymphoblast cell line (IM-9) and the human erythroleukemia cell line (HEL) were also obtained from ATCC (CC9 159 and TIB 180 resp.). These cell lines were propagated in RPMI 1640 containing 10% FCS. 24 hours prior to an experiment, cells were placed in medium containing 0.2% BSA and 0.4% FCS. Incubations were run for 30 hours in this medium.

Electrophoresis and Western Immunoblotting

Cell pellets from adrenal glomerulosa, HEL cells and normal human platelets were lysed in lysis buffer containing PBS (pH 7.4), Triton X-100 1%, phenylmethylsulfonyl fluoride (PMSF) (1 mM), leupeptin (50 μM) and sodium dodecyl sulfate (SDS) 0.1%. Lysates were centrifuged at 10,000 Xg for 10 minutes. An aliquot of the supernatant (cytosol) was saved for protein estimation and the remainder saved at -70°C for Western Blot analysis.

SDS polyacrylamide gel electrophoresis (10% running gel, 4% stacking gel) was performed according to the method of Laemmli (10). For Western blotting, gels were equilibrated in transfer buffer (35 mM Tris base, 192 mM glycine and 20% methanol, pH 8.3) and then transferred to nitrocellulose (Hybond, Amersham) as described by Towbin, et al. (11), in a semi-dry

polyblot apparatus (American Bionetics, Inc.) for 40 minutes at 2.5 mA per sq.cm. of gel. The nonspecific sites were blocked with PBS containing 10% of fetal calf serum at 4°C overnight. The membranes were then washed twice with PBST (PBS + 0.05% Tween-20) and incubated with primary antibody in PBST containing 1% BSA and 20% v/v/ fetal calf serum for 2 hours at room temperature. A polyclonal antibody against porcine 12-LO raised in rabbit (12) was used at 1:600 dilution. In addition, a polyclonal antibody was utilized which was raised to a specific 12-LO peptide with the sequence of amino acids 646-662 of the porcine leukocyte 12-LO sequence (13). This antiserum was used at 1:100 dilution. The washed membranes were then incubated for 1 hour with second antibody (goat anti-rabbit) conjugated with alkaline phosphatase (1:5000, Promega Corp., CA). Detection was either by color development using substrate mixture (NBT and BCIP from Promega) or by chemiluminescence using CSPD substrate and the Western-Light Chemiluminescent detection system (Tropix, Inc., Bedford, MA). Non-specific binding was evaluate using normal rabbit serum. Western blots were quantitated using a computerized video densitometer (Applied Imagining Lynx DNA vision, Santa Clara, CA) and values expressed as arbitrary optical density units.

Assay of 12-LO and 15-LO Products

These assays were performed using a previously published (7, 14) HPLC and RIA technique after extraction on Bond Elut minicolumns.

cDNAs

Recombinant Bluescript plasmid containing the cDNA for human reticulocyte 15-LO (provided by Dr. E. Sigal (U.C. San Francisco, CA)); pUC19 plasmid

containing the cDNA for porcine leukocyte 12-LO was obtained as described previously (14). " Bluescript plasmid containing the cDNA for human platelet 12-LO (provided by Professor Bengt Samuelsson, Karolinska Institute, Stockholm, Sweden (15)). The full length cDNA for human 15-LO were prepared by EcoR 1; Sal 1 and Not 1 digestion of the plasmids, respectively.

In Situ Hybridization

Use of discarded human tissue was approved by the appropriate institutional review committees. All procedures were performed in an RNase-free environment. Normal human adrenal gland removed incidentally at surgery was snap-frozen, embedded in OCT compound, cut at 5 μm, and affixed to acid-cleaned slides. The slides were air-dried for 1 minute, and fixed in 4% formalin in phosphate buffered saline for 5 minutes. The slides were either used immediately, or stored in 70% ethanol at 4°C until use. The slides were washed twice in 2X SSC (1X SSC contains 0.15 M NaCl, 0.015 M sodium citrate) for 10 minutes at room temperature, and then were incubated with prewarmed prehybridization solution (4X SSC, 50% formamide, 1X Denhardt's solution, 5% Dextran sulfate, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA) at 37°C for 1 hour. The prehybridization solution was gently aspirated from the slides, and 100 μl of prehybridization solution containing a 1:200-1:2000 dilution of the labeled 12-LO riboprobe solution (prepared as described below) was placed on each slide. The slides were incubated overnight in a humidified chamber at 37°C. After hybridization, the slides were washed at room temperature twice with 2X SSC for one hour, once with 1X SSC for one hour and once with 0.5X SSC for 30 minutes. The slides were then washed with

buffer 1 (100 mmol Tris-HCl, 150 mmol NaCl, pH 7.5) at room temperature for five minutes.

The 12-LO riboprobes was prepared as follows: The Kpn I/BamH I insert was isolated from pUC 19-porcine leukocyte 12-LO cDNA plasmid and inserted into Bluescript (Stratagene, La Jolla, CA). RNA in-vitro transcription was performed with T7 (sense) and T3 (antisense) RNA polymerase and promoters (Stratagene), inserting digoxigenin-labeled rUTP (Boehringer Mannheim, Indianapolis, INC) into the riboprobe according to the manufacturer's instructions. The product was precipitated with LiCl, washed with ethanol, and limited alkaline hydrolysis of the product was then performed for 30 minutes. The final product was dissolved in 100 μl of DEPC treated water. The size of the transcript was checked by agarose gel electrophoresis.

Oligonucleotide Primers and Probes For PCR

β_2 microglobulin oligonucleotides were a gift of Dr. Perrin White (Cornell University Medical College, NY). Other oligonucleotides including human GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer, and were purified by polyacrylamide gel electrophoresis. The sequences of oligonucleotides are listed in Table 1 and were designed based on known gene sequences (13, 15, 16, 17) and selected from regions displaying most divergence between porcine 12-LO and 15-LO sequences (18).

TABLE 1

Primers and Probes for Amplification and Detection

		Sequence (5′ - 3′)	Position
Human 15-LO	5′ Primer	AACTCAAGGTGGAAGTACCGGAG	146 to 168 SEQ ID NO: 1
	3′ Primer	ATATAGTTTGGCCCCAGCCATATTC	453 to 477 SEQ ID NO: 2
	Probe:	AGGCTCAGGACGCCGTTGCCC	306 to 326 SEQ ID NO: 3
Porcine Leukocyte 12-LO	5′ Primer	TTCAGTGTAGACGTGTCGGAG	145 to 165 SEQ ID NO: 4
		ACTGCTGGTTTGTGAAACTGCGC	177 TO 198 SEQ ID NO: 5
	3′ Primer	ATGTATGCCGGTGCTGGCTATATTTAG	451 to 477 SEQ ID NO: 6
		AATTAACCCATCCTTCCAGTTAC	428 to 450 SEQ ID NO: 7
	Probe	TCAGGATGCGGTCGCCCTCCAC	301 to 322 SEQ ID NO: 8
human GAPDH	5′ Primer	CCCATCACCATCTTCCAGGAG	211 to 231 SEQ ID NO: 9
	3′ Primer	GTTGTCATGGATGACCTTGGC	475 to 495 SEQ ID NO: 10
	Probe	CTAAGCAGTTGGTGGTGCAGG	446 to 466 SEQ ID NO: 11
Human platelet 12-LO	5′ Primer	GATGATCTACCTCCAAATATG	472 to 492 SEQ ID NO: 12
	3′ Primer	CTGGCCCCAGAAGATCTGATC	610 to 630 SEQ ID NO: 13
	Probe	GTTTGAGGGCCATCTCCAGAGC	544 to 565 SEQ ID NO: 14

Amplification of Reverse Transcribed RNA
Using the Polymerase Chain Reaction (RT-PCR)

Total RNA from both human adrenal glomerulosa tissue and cultured cells was extracted with guanidium thiocyanate-phenol-chloroform using RNAzol (Cinna/Biotex Laboratories International, Inc., Texas). Poly (A)+RNA was purified b oligo (dT) cellulose chromatograph column (5 prime —> 3 Prime, Inc. West Chester, Pennsylvania). 1 μg of total RNA

or mRNA was mixed with the PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 200 μM of each of the four deoxynucleotide trisphosphates, 25 pmole each of 5′ and 3′ primers 5′TTCAGTGTAGACGTGTCGGAG3′ (SEQ ID NO: 4) and 5′ATGTATGCCGGTGCTGGCTATATTTAG3′ (SEQ ID NO: 6), 2 units of Avian Myeloblastosis Virus reverse transcriptase (20 U/ul, Life Sciences, St. Petersburg, FL) and 2.5 units Taq polymerase (Perkin Elmer Cetus), in a final volume of 50 μl. In some reactions, 5 pmole of each 5′ and 3′ primers of β_2 microglobulin or GAPDH were added as an internal standard. The samples were placed in a thermal cycler at 37°C for 8 minutes for the reverse transcriptase reaction to proceed. Then conditions used for PCR were a denaturation step at 94°C for 1 minute, annealing at 50°C for 2 minutes, and extension at 72°C for 2 minutes for 25-30 cycles. Blank reactions with no RNA template, or with no reverse transcriptase were carried out through the RT and PCR steps. The human 15-LO cDNA, porcine leukocyte 12-LO cDNA and human platelet 12-LO cDNA amplifications were carried out by mixing 2-5 ng of cDNA in 50 μl volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each of the four deoxynucleotide triphosphates, 25 pmole of 5′ and 3′ primers, and 2.5 U of Taq polymerase. The conditions for PCR were the same as described before.

Gel Analysis and Blot Hybridization

20 μl aliquots of the PCR products were subjected electrophoresis in a 1.8% agarose gel in Tris acetate-EDTA buffer. AFter staining with ethidium bromide and photographing, the gel was transferred onto Zeta probe membrane (Bio-Rad, Richmond, CA) by

capillary blotting. The oligonucleotides used as probes were labeled at the 5′ end using [γ-^{32}P]-ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and hybridized with membrane overnight in 6X SSC, 9.5% Blotto, 7% SDS at 42°C. Membranes were washed once in 6X SSC at room temperature for 15 minutes and then once at 60°C for 15 minutes. The washing conditions were worked out to distinguish between the PCR products of human 15-LO from those of porcine leukocyte 12-LO. The filters were exposed to Kodak X-ray film with an intensifying screen at -70°C. Blots were quantitated using a computerized video densitometer.

Direct DNA Sequencing of PCR Product

(a) PCR amplification: RNA was reverse transcribed as follows: the reaction mixture contained porcine leukocyte 12-LO complementary primer 5′ATGTATGCCGGTGCTGGCTATATTTAG3′ (SEQ ID NO: 6), dNTP and 2 μg of RNA in a final volume of 9 μl. The mixture was heated to 80°C for 5 minutes, cooled to 37°C. 2 units of AMV reverse transcriptase was added and maintained for three minutes at 37°C. Then an additional 2 units of AMV reverse transcriptase was added, the sample was heated to 95°C to denature, and then amplified for 40 cycles by PCR as described before. The PCR product was analyzed by hybridization. To obtain sufficient amount of PCR product for sequencing, 1 μl of total product of the PCR reaction was used as a template for secondary PCR amplification with 5′ and 3′ primers (145-165 and 451-477) primers for U937 cells; (177-198 and 428-450) primers for human adrenal. The reaction conditions were as described as before except that 30 cycles were used.

(b) Preparation of DNA for sequencing: The products of secondary PCR were purified by electrophoresis on nondenaturing 8% polyacrylamide gel. The isolated DNA fragment was directly used for sequencing.

(c) Sequencing: Two approaches were used for sequencing:

(i) Sequencing reaction of purified PCR product of U937 cells was set in the presence of 0.5% NP-40 detergent (19) and [γ^{32}-P] ATP labeled porcine leukocyte 12-LO oligonucleotides 145-165, 451-477 and 301-322. DNA sequencing reactions were performed by the dideoxynucleotide chain termination method (20) using Sequenase (United States Biochemicals, Cleveland, Ohio), sequencing in both directions with 5' primer and 3' primers.

(ii) Sequencing reaction of PCR products of human adrenal glomerulosa tissue and U937 cells was performed by a cycle sequencing method of AmpliTaq DNA polymerase (21) with a cycle sequence kit (Perkin Elmer Cetus, Norwalk, CT). 15 ng of human adrenal PCR product and 2 pmole of [γ-^{32}P]-ATP labeled porcine leukocyte 12-LO oligonucleotide (177-198 or 428-450) were used. The cycling program was 1 minute at 95°C, and 1 minute at 60°C for 20 cycles.

Amplification of Leukocyte Type 12-LO From Human DNA

Human genomic DNA was isolated from leukocyte as described (22). The PCR amplification conditions were as described before except that the reaction mix contained 0.5 μg human DNA and also 0.1 u of perfect match polymerase enhancer (Strategene, LaJolla, CA).

Initial PCR was performed with porcine leukocyte 12-LO primers 145-165 and 451-477. Primers 177-198 and 428-450 were used in secondary PCR. The product was analyzed by 1.2% agarose gel and by hybridization with leukocyte type 12-LO probe.

Northern Blot Analysis

Total RNA from normal human adrenal glomerulosa cells was electrophoresed in a 1.2% agarose, 0.6M formaldehyde gel at 120V for 3-4 hours, and transferred onto Zeta-probe membrane by capillary blotting for 20-24 hours (23). The RNA marker (0.24-9.5 kb RNA ladder BRL, Gaithersburg, MD) was stained in ethidium bromide. Porcine leukocyte 12-LO oligonucleotide 301-322, or porcine leukocyte 12-LO cDNA was used as hybridization probes. The oligonucleotide probe was end labeled with [γ-^{32}P]-ATP as described earlier. A Sal 1 fragment of pUC19 - porcine leukocyte 12-LO cDNA was excised and labeled by [α-^{32}P] dCTP (3000 Ci/mmole) with the random prime DNA labeling kit (Molecular Biology Boehringer Mannheim, Indianapolis, IN). The unincorporated [^{32}P]-ATP or CTP was removed by chromatography on a Sephadex G-50 column. Membranes were hybridized at 37°C for 16 hours to 20 hours in a solution containing 50% formamide, 1 M NaCl, 1% SDS, 10% Dextran Sulfate, 100 μg/ml of herring sperm DNA< and then washed with 2X SSC/0.1% SDS at room temperature. The membranes were exposed to Kodak X-ray film with two intensifying screens at 70°C.

3. Results of Experimental Procedures

Immunodetection and Regulation of Leukocyte Type 12-LO Protein in Cultured Human Adrenal Glomerulosa Cells

Figure 1 shows the specific expression of porcine leukocyte type 12-LO in normal human adrenal glomerulosa cell cytosols using immunoblotting with a polyclonal antibody to a synthetic porcine leukocyte 12-LO peptide. A 72kD band is seen in lane 2 which has authentic partially purified 12-LO from porcine leukocytes. Lanes 4 and 5 which have adrenal glomerulosa cell cytosol fractions also have this clear band with a molecular weight of 72kD, which is identical to the reported molecular weight of porcine leukocyte 12-LO (12). To confirm that the antibody does not cross react with the human platelet form of 12-LO, immunoblots were performed with lysates prepared from HEL cells as a negative control (lane 3). There was no 72kD band in these cells. Hence this antibody does not cross react with the human platelet form of 12-LO.

Figure 1 also shows the effect of AII (10^{-7} M) treatment for 50 hours (lane 5) on the expression of the 12-LO protein in normal human adrenal glomerulosa cells as assessed by Western immunoblotting. AII increased the expression of 12-LO more than two-fold over basal (lane 4) as determined by video densitometric analysis. Similar results were also obtained using a polyclonal antibody to purified porcine leukocyte 12-LO enzyme (results not shown).

Detection of hl 12-LO mRNA in Human Adrenal Glomerulosa Cells By Northern Blot Analysis

Northern analysis was performed to evaluate mRNA expression in human adrenal glomerulosa cells. As shown in Figure 2, a clear band of approximately 4 kb

size was found in two samples of adrenal glomerulosa cells using the [^{32}P]-labeled leukocyte 12-LO probe.

In Situ Hybridization

A strong hybridization signal was seen with the antisense 12-LO riboprobe, primarily in the zona glomerulosa of the adrenal cortex, although, upon prolonged incubation with the chromogen, a very slight positive reaction could also be detected in the other layers of the cortex as well (Figure 3). The negative controls, including the sense probe, failed to give a signal.

RT-PCR Analysis of mRNA From Adrenal and Other Cell Types

RT-PCR assay was used to confirm whether porcine leukocyte type 12-LO was present in human cells. PCR amplification of the region at the most divergent area of amino acid sequence of the porcine leukocyte 12-LO and human 15-LO was selected. The size of the amplified fragment is 333 bp for both of the porcine leukocyte 12-LO and human 15-LO. Using RNA samples from the human cells, the 333 bp amplified product of the leukocyte type 12-LO could not be seen in an ethidium bromide stained gel, but was detected specifically by autoradiography of a blot hybridized with a porcine leukocyte 12-LO oligonucleotide probe.

Since the amino acid sequences of porcine leukocyte 12-LO and human 15-LO are highly homologous, the porcine leukocyte 12-LO cDNA probe could not distinguish the 33 bp amplified products corresponding to porcine leukocyte 12-LO or to human 15-LO (data not shown). Moreover, human 15-LO oligonucleotide and porcine leukocyte 12LO oligonucleotide probes can cross hybridize to the 12-LO or 15-LO amplified product, respectively using 12-LO or 15-LO cDNA as templates of amplification. A

condition was established to distinguish the 333 bp PCR amplified products of 12-LO and 15-LO by raising the washing temperature of the hybridized membranes to 60°C. Figure 4 indicates that at 60°C, no hybridization is seen between the porcine leukocyte 12-LO oligonucleotide probe and the 15-LO PCR product. However, hybridization is observed with the 12-[LO PCR product (Figure 4A). No hybridization was seen between human 15-LO oligonucleotide probe and 12-LO PCR product, but hybridization with the 15-LO PCR product was observed (Figure 4B). Tests were also conducted to determine human 15-LO cDNA could be amplified by leukocyte 12-LO primers. However, no fragment was detected either in ethidium bromide stained gel analysis or in Southern blot using a human 15-LO probing analysis (data not shown). These results suggested that the leukocyte 12-LO primers did not cross amplify human 15-LO in our experimental conditions and are therefore specific to porcine leukocyte 12-LO.

Based on these results, analysis for porcine leukocyte type 12-LO in human adrenal glomerulosa cells and in several other human cell lines were conducted by the RT-PCR assay. A 333 bp amplified product derived from transcripts of porcine leukocyte type 12-LO was detected after 30 cycles of amplification using total RNA from human normal adrenal glomerulosa or mRNA or total RNA from human U937 cells (Figure 5). Two negative controls for the RT-PCR were tubes without template RNA and without reverse transcriptase. Amplified products with human 15-LO cDNA template and with porcine 12-LO cDNA template were used as a negative control and positive control in the Southern blot hybridization, respectively (Figure 5). Further support that U937

cells contain an active 12-LO was that U937 cells only synthesize 12(S)-HETE (59 pg/10^6 cells, n=5) with no synthesis of 15 (S)-HETE (levels below HPLC and RIA detection limit).

In addition, RT-PCR experiments were performed with other human cells such as HEL cells and IM-9 cells (Figure 6). A 404 bp amplified product of β_2 microglobulin which used as a control for RNA amplification efficiency could be seen in HEL cells or in IM-9 cells in an ethidium bromide stained gel (Figure 6A). In contrast, when the blot was hybridized with the leukocyte type 12-LO probe, the 333 bp amplified 12-LO was not detected in HEL cells or in IM-9 cells, but was detected again (see Figure 6C) in U937 cells as shown in Figure 5. These results suggest that no leukocyte type of 12-LO is expressed in these negative control cell types, HEL cells and IM-9 cells. The expression of the human platelet 12-LO was also studied in these human cells by RT-PCR amplification with human platelet primers as well as GAPDH primers (Table 1). The 284 amplified product of GAPDH which used as a control for RNA amplification efficiency was similar in al RNA samples (Figure 6B). The expected 159 bp hybridization band of platelet type 12-LO probe was seen only in HEL cells which expresses the platelet type 12-LO. However, no 159 bp hybridization band was seen in human adrenal glomerulosa cells or in U937 cells (Figure 6D). These results suggest that human glomerulosa cells and U937 cells do not express the platelet type 12-LO.

Figure 7 shows regulation of 12-LO mRNA levels by AII determined by RT-PCR. Total RNA was extracted from cultured adrenal glomerulosa cells that were incubated alone or with 10^{-7}M AII for 24 hours. RNA

samples were amplified for 25 cycles with primers amplifying porcine 12-LO. All reactions in the experiment also contained primers amplifying human GAPDH. Controls without RNA or with RNA pretreated with RNAase were simultaneously run. The position of the specific products are indicated by arrows. 284 bp and 333 bp represent amplified products of human GAPDH and porcine leukocyte 12-LO respectively. Panel A is the autoradiogram of the blot hybridized with oligonucleotide probe specific for the porcine 12-LO gene. Panel B is the autoradiogram of the same blot subsequently hybridized with oligonucleotide probe for the GAPDH. Lanes 1 and 4 are glomerulosa cells in the control incubation. Lanes 2 and 5 are glomerulosa cells incubated with 10^{-7}M AII. Samples in lanes 4 and 5 were treated with RNase A prior to the RT-PCR. Lane 3 is without RNA. These results show that 12-LO gene is present in human glomerulosa and monocytes and that the RNA is upregulated by AII.

Furthermore, amplified genomic DNA from human leukocyte nuclei shows that the gene size in the segment amplified (1 Kb) was substantially larger than the expected size in the same region in the porcine gene.

Figure 8 shows identical procedures as outlined previously for protein expression that AII can increase 12-LO protein expression in human aortic smooth muscle cells. The increase of expression was seven fold as measured using a computerized video densitometric system. Figure 9 shows expression of 12-LO RNA in human vascular smooth muscle cells using a similar RT-PCR procedure. Lane 5 shows expression of the expected 333 base pair 12-LO band in human vascular smooth muscle cells, while lane 3 shows an identical RNA band in samples taken from mononuclear

cells. Basal expression of 12-LO in unstimulated smooth muscle cells is below the detection limit of this experiment (lane 7). However, smooth muscle cells stimulated by AII show a marked increase in 12-LO expression (lane 5). Panel B represents an ethidium bromide stain of the RT-PCR experiment showing internal marker RNA (B_2 microglobulin) for these experiments (lanes 2, 4, 6). Figure 10 illustrates the stimulatory effect of AII at 10^{-9} and 10^{-8} M on 12-HETE synthesis and release from human aortic smooth muscle cells. 12-HETE was assayed by HPLC and specific radioimmunoassay.

Sequencing of PCR Product

The 333 bp PCR amplified product obtained from U937 cell RNA and 274 bp product from human adrenal tissue RNA was purified, sequenced, and found to contain a sequence 2 base pairs different than the published porcine leukocyte 12-LO cDNA in the region spanning nucleotides from 199 to 437 (13) (Table 2).

TABLE 2

Comparison of nucleotide sequences of porcine leukocyte 12-LO (p12-LO) cDNA and human leucocyte (adrenal) 12-LO (hl 12-LO) cDNA at the region between position 199 and 437 of published porcine leukocyte 12-LO cDNA seqeunce (14). 2 nucleotide differences are at position 255 (T/C) and position 267 (C/T).

```
hl 12LO  AAACGGCACCTCCTTCAGGATGACGCGTGGTTCTGCAATTGGATCTCCGTGCAGGGTCCGGGAGCAAACGGG
         |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||| |||
P12LO    AAACGGCACCTCCTTCAGGATGACGCGTGGTTCTGCAATTGGATCTCCGTGCAGGGCCCGGGAGCAAATGGG
         200       210       220       230       240       250       260       270

hl 12LO  GACGAGTTCAGGTTCCCCTGCTACCGCTGGGTGGAGGGCGACCGCATCCTGAGCCTCCCTGAGGGCACTGCC
         |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
P12LO    GACGAGTTCAGGTTCCCCTGCTACCGCTGGGTGGAGGGCGACCGCATCCTGAGCCTCCCTGAGGGCACTGCC
                 280       290       300       310       320       330       340

hl 12LO  CGCACAGTGGTCGATGACCCTCAAGGCCTGTTCAAGAAACACAGGGAGGAGGAGCTGGCAGAGAGAAGGAAG
         |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
P12LO    CGCACAGTGGTCGATGACCCTCAAGGCCTGTTCAAGAAACACAGGGAGGAGGAGCTGGCAGAGAGAAGGAAG
               350       360       370       380       390       400       410

hl 12LO  CTGTATCGGTGGGGTAACTGGAA  (SEQ ID NO. 15)
         ||||||||||||||||||||||
P12LO    CTGTATCGGTGGGGTAACTGGAA  (SEQ ID NO. 16)
             420       430
```

To compare the intron size of human DNA at the region spanning (177-450) with that of porcine leukocyte 12-LO DNA, the human DNA was amplified by PCR. The results in human genomic DNA indicate a PCR product of ≈ 1Kb as compared to the expected 0.64 Kb size for the same region in porcine DNA (24).

4. Summary Regarding hl 12-LO

These results from several experimental approaches provide the first demonstration that another type of 12-LO is expressed in humans in addition to the 12-LO found in the platelet. Initial studies showed that a polyclonal antibody to the leukocyte type of 12-LO revealed a strong band in adrenal glomerulosa lysates with a molecular weight identical to the porcine leukocyte 12-LO. The 12-LO antibody used did not detect a 72 kD band in HEL-cells suggesting that this 12-LO antibody does not cross react with the human platelet 12-LO proteins. Furthermore, Northern analysis using porcine 12-LO oligonucleotide probes shows specific hybridization.

The 15-LO originally cloned from the reticulocyte and found in several human tissues is highly homologous (86% sequence homology) to the porcine leukocyte 12-LO (13). Therefore, it is unlikely that Northern analysis, even under stringent conditions, can with certainty distinguish these two mRNA types. However, the size of the transcript obtained (≈4kb) is larger than the RNA size expected for 15-LO. In addition, a PCR technique was developed which utilized oligonucleotide primers and the appropriate hybridization probes in the most divergent regions of these two cDNA regions. The specificity of the approach was revealed using the 12-LO and 15-LO

cDNA's as templates for amplification. Using this approach and set of conditions, specific amplification of the leukocyte 12-LO and 15-LO could be accomplished. Therefore, the Southern blot hybridization using the leukocyte 12-LO probe provides strong evidence that the band seen is truly a 12-LO, and not the 15-LO product. Additional support that a 12 and not a 15-LO is being detected is that (1) only 12(S)-HETE is stimulated by physiologic concentrations of angiotensin II (10^{-9}M) in adrenal glomerulosa cells (7, 14), and (2) sequence analysis of the amplified segment in U937 and glomerulosa cells gives a sequence much closer to the published porcine leukocyte 12-LO (13).

Several approaches were utilized in the PCR studies to control the specificity of the amplification reaction. First, no amplification for leukocyte type 12-LO was found when the IM-9 cell or HEL cells RNA were utilized as templates. These controls are important since the IM-9 lymphocyte cell does not have 12-LO activity. In addition, the platelet type 12-LO was amplified in HEL cell which is thought to express only the platelet type of 12-LO (15, 18, 25). Furthermore, no amplification was obtained in reactions without RNA templates or reverse transcriptase. Similarly, no band was seen when RNase was added.

It was also found that leukocyte type of 12-LO is expressed in the human mononuclear like cell, U937 and normal monocyte. These results provide additional proof that the leukocyte type of 12-LO is expressed in human tissues. To further substantiate that the PCR reaction is specifically amplifying the product of interest, the 5'-3' ends were sequenced in the amplified sequence from the human monocytic cell

U937. This cell was chosen since it has exclusively 12- and no 15-LO activity, and the amplified product size is identical to that found using the human adrenal RNA. The sequence revealed only a 2 base pair difference from the published sequence for the porcine leukocyte 12-LO, providing additional support that the leukocyte form of 12-LO in humans is similar to that seen in porcine leukocyte.

Several lines of evidence suggest that the sequence obtained from U937 cells and adrenal glomerulosa are not due to cDNA contamination or PCR carryover. The identical sequence is seen in both U937 cells and adrenal glomerulosa despite no amplified product seen in HEL and IM-9 cells which do not appear to express the porcine leukocyte type of 12-LO. Also control experiments using no template show no amplified product. Finally, genomic DNA analysis from human leukocyte using 12-LO primers indicates the presence of a leukocyte type of 12-LO but the intron size is substantially bigger than the expected intron size for the porcine gene.

It is not possible using the human adrenal tissue to completely exclude fasciculata tissue in preparing glomerulosa cells. Based on microscopic morphology and cortisol production, the cultures utilized were approximately 90% pure glomerulosa cells. However, to fully determine whether the 12-LO message is expressed only in the glomerulosa, a sensitive in situ hybridization method using sense and antisense riboprobes for the leukocyte type of 12-LO was utilized. The results revealed a specific signal primarily in the glomerulosa region of the adrenal cortex, suggesting that the zona glomerulosa is one of the major sites of 12-LO message synthesis.

The regulation of the 12-LO protein and gene expression in the glomerulosa cell is not known. Evidence from this application suggests that AII can increase the expression of the 12-LO protein and mRNA. The RNA was increased by nearly 60 fold while protein levels using Western analysis increased over 2 fold by AII. These results provide the first evidence for hormonal regulation of a 12-LO enzyme in a mammalian cell.

Applicants identified for the first time expression and regulation of a new type of 12-LO in a human tissue. hl 12-LO enzyme activation plays a key role in mediating AII action in several tissues including the adrenal, kidney, juxtaglomerular cells and blood vessel (26). Therefore, understanding the regulation of hl 12-LO expression may be an important factor leading to novel mechanisms for treatment of disease states such as hypertension and diabetes, which are characterized by enhanced AII action.

B. REGULATION OF 12-LO ACTIVITY AND EXPRESSION

1. Elevated Glucose and AII Increase 12-LO Activity and Expression

The mechanisms underlying the accelerated atherosclerotic disease associated with diabetes mellitus are not very clear. Prolonged hyperglycemia is considered to be a key factor in leading to diabetic vascular complications since it can alter key biochemical pathways related to glucose metabolism (27). Elucidation of the mechanisms that regulate vascular smooth muscle cell (VSMC) proliferation is crucial to understanding the increased risk of cardiovascular disease in diabetes. It is known that porcine VSMCs (PVSMCs) cultured in high glucose (HG; 25 mM) proliferate at a significantly faster rate and with increased

cell-associated levels of the 12-lipoxygenase (12-LO) product of arachidonic acid, 12-hydroxyeicosatetraenoic acid (12-HETE), than cells cultured in normal glucose (NG; 5.5 mM) (28). Recent studies have implicated the LO enzymes and their products--namely, HETEs and hydroxyoctadecadienoic acids (HODEs)--in the pathogenesis of atherosclerosis (29). It was observed that the 15-LO mRNA and protein colocalize with epitopes of oxidized low density lipoprotein (LDL) (30) and cellular oxidation of LDL can be attenuated by LO inhibitors (31). Furthermore, increased levels of HETEs and HODEs were observed in the aortas of atherosclerotic rabbits (32). HETEs have also been suggested to play a key role in the pathogenesis of diabetic vascular disease, since vessels from infants of diabetic mothers had significantly elevated levels of 15-HETE and decreased formation of vasodilatory prostacyclin (33) and endothelial cells cultured under hyperglycemic conditions produced increased amounts of HETEs (34). Studies also suggest that HETEs have mitogenic properties (35) and can cause SMC migration at concentrations as low as 1 pM (36).

It has been demonstrated that the vasopressor effects of angiotensin II (AII) are enhanced in the diabetic state (37). Increasing evidence also indicates that AII is an important local factor in leading to VSMC hypertrophy and neointimal SMC proliferation (38, 39). It is known (14, 40) that the 12-LO pathway of arachidonic acid plays a key role in AII-induced aldosterone synthesis and adrenal cell proliferation. Furthermore, inhibition of the LO pathway can reduce vasopressor responses to AII (26). This invention implicates the effects of

HG and AII on LO pathway activation in cultured PVSMCs.

A leukocyte type of 12-LO has been purified and cloned from porcine leukocytes (5, 13). However, no studies have addressed whether this form of 12-LO is also present in VSMCs. Furthermore, the factor(s) regulating the expression of 12-LO is not known. Pursuant to this aspect of the invention, it was determined that the 12-LO enzyme is expressed in PVSMCs.

2. Description of Figures 13 to 19

Figure 13 depicts the effect of HG (25 mM) on cell-associated 12- and 15-HETE levels in PVSMCs. HETEs in cell pellets were measured after a 20-minute incubation in serum-free medium. Results are expressed as means ± SE from five experiments performed in duplicate. *, $\underline{P} < 0.01$ v. NG, paired.

Figure 14 depicts the effect of AII (10 nM) on cell-associated 12-HETE levels in NG or HG. Results are expressed as means ± SE from four experiments performed in duplicate. *, $\underline{P} < 0.01$ vs. NG control; **, $\underline{P} < 0.01$ vs. HG control, paired.

Figure 15 depicts specific expression of porcine leukocyte 12-LO protein in PVSMCs. Lanes: 1, partially purified authentic porcine leukocyte 12-LO; 2 and 3, PVSMC cytosols. (A) With porcine leukocyte 12-LO antibody. (B) With 12-LO antibody preincubated with authentic 12-LO.

Figure 16 depicts expression of porcine leukocyte-type 12-LO (72 kDa) in PVSMCs cultured in NG or HG and after AII (100 nM) treatment for 45 hours. Equal amounts of protein were electrophoresed and blots were probed with a specific antibody to

porcine leukocyte 12-LO. Similar results were found in three separate experiments.

Figure 17 identifies porcine leukocyte-type 12-LO mRNA in PVSMCs by reverse transcriptase PCR and its regulation by HG. One microgram total RNA samples were amplified for 25 cycles with porcine leukocyte 12-LO primers. Positive control with 12-LO cDNA is shown in the rightmost lane. Similar results were found in three separate experiments. (A) Hybridized with 12-LO oligonucleotide probe. (B) Same membrane hybridized with GAPDH probe.

Figure 18 is a Southern blot showing that the 333-bp specific amplified products of porcine leukocyte-type 12-LO are detected in PVSMCs cultured in both NG as well as HG. In addition, cells cultured in HG had a 20-fold increase in expression of the 12-LO PCR amplified product. OD of the 12-LO band in arbitrary units: HG, 3092; NG, 137. OD of GAPDH band (284 bp): HG, 1247; NG, 1115. Figure 18 shows that 12-LO mRNA expression (333 bp) was much greater in HG (with little basal expression in NG). In addition, AII (100 nM) treatment for 24 hours caused a significant 3- to 4-fold increase in expression in both NG and HG according to the following OD readings: 12-LO band, HG control, 1072; HG AII, 3000; NG control, 40; NG AII, 303; GAPDH band, HG control, 1214; HG AII, 1293; NG control, 667; NG AII, 1168. Amplified products of 15-LO were not detected with specific PCR primers and probes for 15-LO (data not shown). 0.5 μg of mRNA was amplified for 25 cycles with leukocyte 12-LO primers A, hybridization with 12-LO probe, B, hybridization with GAPDH probe.

Figure 19 depicts reverse-phase HPLC analysis of products of cold arachiodonate metabolism by sonicated PVSMCs grown in NG (C and D) or HG (E and F). Detection was at 237 nm. Arrows a and b indicate positions of 15- and 12-HETE, respectively. (A) Retention time of authentic cold 15- nd 12-HETE (17.2 and 18.0 minutes, respectively). (B) Tracing of enzyme blank. (C and E) Basal (control) activity in NG and HG, respectively. (D and F) Activity with 10-minute AII (100 nM) treatment in NG and HG, respectively. Results shown are representative of three experiments.

Figure 20 illustrates the regulation of 12-LO protein expression by AII in human adrenal glomerulosa cells.

3. Experimental Procedures

Materials and Methods

Culture of PVSMCs. Primary cultures of PVSMCs were obtained as described (28). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing NG (5.5 mM) and 10% fetal calf serum (FCS). For studies under hyperglycemic conditions, the cells were cultured for at least two passages in DMEM HG (25 mM) before use. Controls for osmolality were cells grown for at least two passages in NG/10.5 mM mannose.

Measurement of LO Products 12- and 15-HETE. Serum-starved (24 hours) confluent cells in NG or HG in 100-mm dishes were placed in medium containing 0.2% bovine serum albumin (BSA) and preincubated for 20 minutes at 37°C, with LO or cyclooxygenase inhibitors added during the last 10 minutes. AII (Peninsula Laboratories) was then added and cells were incubated for an additional 10 minutes. The

reaction was terminated by cooling on ice. The HETE(s) in the supernatants was extracted as described (41). Cell pellets were first deacylated to release the cell-associated esterified HETEs by treating them with 1.5 ml of 0.2 M NaOH in methanol and 50 μM n-propyl gallate (an inhibitor of nonspecific fatty acid oxidation (32)) under N_2 for 45 minutes. The solution was then diluted to 15% methanol and extracted (41). The LO products in the supernatants as well as cell extracts were quantitated by a specific RIA (14). The 12-HETE RIA is specific for 12(S)-HETE with <0.1% crossreactivity with 12(R)-HETE. The identity of the HETEs was confirmed by comingration with authentic cold 12(S)- and 15(S)-HETE using a gradient reverse-phase HPLC system (14).

Measurement of LO Activity. Confluent PVSMCs in NG or HG medium were harvested, suspended in 1 ml of Tris-HCl buffer (25 mM; pH 7.7), and then sonicated on ice. The assay mixture contained in 1.0 ml, 450 μl of enzyme (sonicate), 400 μl of Tris buffer, and 100 μl of glutathione (9.5 mM). An enzyme blank was run simultaneously. The reaction was started at 37°C with 50 μl of sodium arachiodnate (160 μM; NuCheck Prep, Elysian, MN). After a 10 minute incubation, the reaction was stopped with 2 ml of isopropanol/1.2% acetic acid followed by 2 ml of chloroform. The lower organic layer was filtered and subjected to HPLC to detect HETEs. Peak heights were quantitated with a Shimazu CR5A integrator.

Incubations for 12-LO Protein or mRNA Expression. Serum-starved PVSMCs in NG or HG in 150-mm dishes were placed in DMEM/Hepes NG or HG containing 0.2% BSA and 0.4% FCS without antibiotics, alone, or with AII. At the end of the incubation,

cell pellets were processed for Western blotting or RNA extraction as described below.

Electrophoresis and Western Immunoblotting. Cell pellets were lysed in buffer containing phosphate-buffered saline (pH 7.4), 1% Triton X-100, 50 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.1% SDS. Lysates were centrifuged at 10,000 x g for 10 minutes and supernatants were subjected to electrophoresis and Western blotting as described (40). A polyclonal antibody against porcine leukocyte 12-LO (12) (generous gift from T. Yoshimoto, Tokushima University, Tokushima, Japan) was used at 1:600 dilution. Western blots were quantitated with a computerized video densitometer and values were expressed as arbitrary optical density (OD) units. For antibody blocking studies, the 12-LO antibody (4 μl) was incubated with or with 30 μl (16 μg of protein) of partially purified 12-LO enzyme (25-50% ammonium sulfate fraction of porcine leukocyte cytosol (42)); gift from T. Yoshimoto] ion 500 μl of tris-buffered saline (pH 7.4) containing 1 mg of BSA per ml for 2.5 hours at room temperature. The blocked and free antibodies were then diluted 1"600 and used to incubate the blots containing the PVSMC cytosol samples.

cDNAs. Recombinant Bluescript plasmid containing the cDNA for human reticulocyte 15-LO was kindly provided by E. Sigal (University of California, San Francisco). pUC19 plasmid containing the cDNA for porcine leukocyte 12-LO (13) was a generous gift from T. Yoshimoto. The full-length 15-LO cDNA and cDNA for porcine leukocyte 12-LO were prepared by EcoRI and Sal I digestion of the plasmids, respectively.

Oligonucleotide Primers and Probes for PCR. All the oligonucleotides including human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer and purified by polyacrylamide gel electrophoresis. The sequences of oligonucleotides (Table 3) were designed based on known gene sequences (13, 16, 17) and were selected from regions displaying most divergence between porcine 12-LO and human 15-LO sequences (18) because of their close homology.

TABLE 3

Primers and Probes for Amplification and Detection

Position		Sequence (5′ - 3′)	
Porcine Leukocyte 12-LO	5′ Primer	TTCAGTGTAGACGTGTCGGAG	145 to 165 SEQ ID NO: 4
	3′ Primer	ATGTATGCCGGTGCTGGCTATATTTAG	451 to 477 SEQ ID NO: 6
	Probe	TCAGGATGCGGTCGCCCTCCAC	301 to 322 SEQ ID NO: 8
Human 15-LO	5′ Primer	AACTCAAGGTGGAAGTACCGGAG	146 to 168 SEQ ID NO: 1
	3′ Primer	ATATAGTTTGGCCCCAGCCATATTC	453 to 477 SEQ ID NO: 2
	Probe:	AGGCTCAGGACGCCGTTGCCC	306 to 326 SEQ ID NO: 3
human GAPDH	5′ Primer	CCCATCACCATCTTCCAGGAG	211 to 231 SEQ ID NO: 9
	3′ Primer	GTTGTCATGGATGACCTTGGC	475 to 495 SEQ ID NO: 10
	Probe	CTAAGCAGTTGGTGGTGCAGG	446 to 466 SEQ ID NO: 11

Amplification of Reverse-Transcribed RNA or PCR. PVSMC total RNA was extracted with guanidium thiocyanate/phenol/chloroform using RNAzol

(Cinna/Biotecx Laboratories, Friendswood, TX). Poly(A)+ RNA was purified by oligo(dT)-cellulose column chromatograph (5 Prime —> 3 Prime, Inc.). One microgram of total RNA or 0.5 μg of RNA was mixed with the PCR buffer (10 mM Tris-HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.001% gelatin), 200 μM each of dNTP, 25 pmole of each of 5′ and 3′ primer, 2 units of avian myeloblastosis virus reverse transcriptase (20 units/μl; Life Sciences, St. Petersburg, FL) and 2.5 units Taq polymerase (Perkin Elmer/Cetus) in a final volume of 50 μl. In all reactions, 5 pmole of each 5′ and 3′ primer of GAPDH was added as an internal standard to control for RNA quantity and amplification efficiency. The samples were placed in a thermal cycler at 37°C for 8 minutes for the reverse transcriptase reaction to proceed. Then conditions used for PCR were a denaturation step at 94°C for 1 minute, annealing at 50°C for 2 minutes, and extension at 72°C for 2 minutes for 25 cycles. Blank reactions with no RNA template, or with no reverse transcriptase were carried out through the reverse transcription and PCR steps. RNA samples from human erythroleukemis (HEL) cells, which do not express the porcine leukocyte 12-LO, were run as negative controls. For positive controls, the human 15-LO cDNA and porcine 12-LO cDNA amplifications were carried out.

Gel Analysis and Blot Hybridization. The PCR products were electrophoresed on 1.8% agarose gels followed by capillary blotting onto Zeta-Probe (Bio-Rad) membrane. Porcine 12-LO, human 15-LO, and human GAPDH oligonucleotides used as probes were labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) and hybridized with membrane overnight in 6X standard

saline citrate (SSC)/0.5% non-fat dry milk/7% SDS at 42°C. Membranes were washed once in 6X SSC at room temperature for 15 minutes and then once at 60°C for 15 minutes. These washing conditions were developed to distinguish between the PCR products of human 15-LO from those of porcine leukocyte 12-LO. After autoradiography, blots were quantitated by using a computerized video densitometer.

4. Results

Effect of Glucose on Immunoreactive Cell-Associated and Released HETE Levels. Figure 13 shows that PVSMCs cultured in HG (25 mM) had significantly greater levels of cell-associated 12- as well as 15-HETE than those cultured in NG (5.5 mM). In contrast, cell associated 12-HETE levels in mannitol-grown cells were not significantly different from those in cells cultured in NG (1493 ± 281 pg per 10^6 cells in NG vs. 1386 ± 232 pg in mannitol). HG did not have any significant effect on the levels of released HETEs (data not shown).

Effect of AII on Immunoreactive Cell-Associated and Released HETEs. AII significantly increased cell-associated 12-HETE levels in cells cultured in HG only (Figure 14). However, AII at concentrations ranging from 100 nM to 100 pM did not increase cell-associated 12-HETE levels in cells cultured in NG. Cell-associated 15-HETE levels were not significantly increased by AII either in HG [basal 15-HETE, 2722 ± 225 pg per 10^6 cells; AII (10 nM), 2928 ± 188 pg] or in NG (data not shown).

Table 4 shows that AII treatment for 10 minutes causes a dose-dependent increase in released 12- and 15-HETE levels in NG. AII at 2 nM selectively increased only 12-HETE. AII (100 nM) also stimulated released 12- and 15-HETE levels in HG (Table 5). The

specific LO inhibitor baicalein (10 μM) (Biomol, Plymouth Meeting, PA) significantly reduced the AII-induced release of 12- and 15-HETE in HG without altering basal HETE release (Table 5). In addition, baicalein also significantly reduced the increase in cell-associated 12-HETE formation by AII in cells grown in HG [2145 ± 90 (basal) vs. 2760 ± 160 (AII) vs. 2004 ± 114 (AII + baicalein) pg per 10^6 cells; P <0.05]. The cyclooxygenase inhibitor meclofenamate (Sigma), however, attenuated AII-induced release only of 15-HETE and not of 12-HETE.

TABLE 4

Dose-dependent Effects of AII on Released 12- and 15-HETE Levels in PVSMCs Grown in NG

	12-HETE, pg per 10^6 cells	15-HETE, pg per 10^6 cells
Basal	119 ± 25	126 ± 10
AII (0.2 nM)	108 ± 27	119 ± 22
AII (2 nm)	171 ± 30*	160 ± 40
AII (20 nM)	239 ± 21**	199 ± 14**
AII (200 nM)	248 ± 32**	220 ± 30**

12- and 15-HETE levels were measured by a specific RIA of cell supernatants after a 10-minute treatment with or without AII. Results are expressed as means ± SE from three to six experiments run in duplicate. *, P < 0.05 vs. basal; **, P <0.01 vs. basal.

TABLE 5

Effect of LO and Cyclooxygenase Blockers on AII-Induced HETE Released in PVSMCs Grown in HG

	Released 12-HETE, pg per 10^6 cells	Released 15-HETE, pg per 10^6 cells
Basal	120 ± 10	138 ± 15
AII (100 nM)	190 ± 28*	196 ± 18*
AII + baicalein (10 μM)	141 ± 26**	144 ± 11**
AII + meclofenamate (1 μM)	170 ± 19*	149 ± 15**
Baicalein (10 μM)	129 ± 12	127 ± 17

Results are expressed as means ± SE from three experiments. Incubations and measurement of immunoreactive released HETEs in the supernatants were performed as described. *, P <0.05 vs. basal; **, P <0.05 vs. AII.

Regulation of Porcine Leukocyte-Type 12-LO Protein and mRNA by AII and HG. The 12-LO protein was identified by immunoblotting with a specific antibody to the porcine leukocyte 12-LO. A distinct band was detected with a molecular mass of nearly 72 kDa (Figure 15A, upper band), which is the reported molecular mass of the porcine leukocyte-type 12-LO (12). In addition, antibody blocking studies were performed to confirm the identity of the band. Immunoprecipitation of the 72-kDa band obtained with authentic porcine leukocyte 12-LO enzyme (lane 1) as well as PVSMC cytosols (lanes 2 and 3) could be completely prevented by using antibody that had been pretreated with partially purified porcine leukocyte 12-LO enzyme (Figure 15B). Furthermore, one or two

lower bands previously visible in the Western blots were also eliminated in the blocking studies. The source of these bands is not clearly known at the present time.

Basal 12-LO enzyme (72 kDa) expression is increased nearly 5-fold in PVSMCs cultured in HG (Figure 16). In addition, AII (100 nM) at 45 hours caused a marked stimulation (2- to 3-fold) of 12-LO expression in NG as well as HG (Figure 16). The 12-LO antibody did not detect any 72-kDa bands with lysates from human platelets and HEL cells (results not shown). We also probed the blots with an antibody to human 15-LO (gift from E. Sigal) and could not detect any bands near 70 kDa, the reported molecular mass for 15-LO (data not shown).

To evaluate the expression and regulation of 12-LO mRNA in PVSMCs, we used a reverse transcriptase PCR assay because of the very low expression of this mRNA. The size of the PCR amplified fragment is 333 bp for both 12- and 15-LO. Specific conditions to distinguish leukocyte 12-LO from human 15-LO were achieved by increasing stringency and raising washing temperature of hybridized membranes to 60°C. PCRs were run at 25 cycles to maintain amplification in a linear range.

Figure 20 shows the effect of AII (10^{-7}M) on the expression of the 12-LO protein in normal human adrenal glomerulosa cells as assessed by Western immunoblotting. AII increased the expression of 12-LO (Figure 20A) approximately two-fold over basal as determined by densitometric analysis (Figure 20B). Thus, the 12-LO protein is present in cultured human glomerulosa cells as seen using an antibody against a porcine leukocyte 12-LO. Furthermore, the 12-LO protein expression is

increased in cells cultured in the presence of AII for 30 hours. A: shows immunoblot of data while B: represents a densitometric analysis of the data in A.

5. Summary as to 12-LO Activity and Expression

Certain 12- and 15-LO enzymes have recently been isolated and their cDNAs were cloned. 15-LO was cloned from human reticulocytes (16). One form of 12-LO was cloned from human platelets and HEL cells (15, 18, 43). 12-LO has also been identified and cloned from porcine leukocytes (5, 13) and bovine tracheal epithelial cells (44, 45). Human platelet and porcine leukocyte 12-LO enzymes share only 65% amino acid identity (15, 18, 43). In contrast, the leukocyte-type 12-LO shows nearly 87% homology with the 15-LO sequence (13). In the present study, it was shown that PVSMCs express the porcine leukocyte-type 12-LO, since a specific antibody to the leukocyte 12-LO immunoprecipitated a protein with a molecular mass similar to that of the porcine leukocyte 12-LO. Furthermore, using a polyclonal human 15-LO antibody, no specific band was seen around 70 kDa. A specific reverse transcriptase PCR technique was used to evaluate 12-LO mRNA expression in PVSMCs. Using the porcine leukocyte 12-LO primers and probe, the expected 333-bp amplified product was detected. However, no clear product was found with the 15-LO primers and probe. It is possible that the 15-HETE made by these cells, in part, arises from the 12-LO pathway or from the cyclooxygenase pathway since the data shows that cyclooxygenase inhibitors can block release of only 15-HETE. However, the results do not completely exclude the possibility that PVSMCs also express a type of 15-LO that may differ sufficiently in homology with the human 15-LO

so as not to be detected with the human 15-LO antibody or probe.

It has been recently demonstrated that PVSMCs cultured in HG proliferate faster and have greater total cellular protein content than those in NG (28). Similar results were also observed in rat VSMCs (46). The results of the present study indicate that PVSMCs cultured chronically in HG synthesize greater amounts of the 12-LO product 12-HETE and have increased 12-LO activity as compared to cells cultured in NG. These effects were not simply due to the hyperosomolar effects of HG since equimolar concentrations of mannitol did not result in excess PVSMC cell growth (28) or increased 12-LO product formation. Although 12-HETE release was not increased in these cells cultured chronically in HG, recent evidence shows that acute HG treatment (24-48 hours) can stimulate levels of released 12-HETE.

Previous studies showed that glucose can increase 12-HETE levels in rat islets (47). Very few studies have evaluated changes in gene expression by HG. Very recently, HG was shown to increase transforming growth factor α and fibroblast growth factor mRNAs in rat VSMCs (48). This aspect of the invention includes the discovery that HG upregulates 12-LO mRNA and protein in PVSMCs.

It also includes the observation that AII specifically increased cell-associated 12-HETE concentrations in cells cultured in HG. Apparently, the 12-LO pathway may be specifically involved in mediating effects of AII in VSMCs when exposed to chronically high levels of glucose as in diabetes. Another aspect of the invention is the discovery that AII can directly increase 12-LO mRNA and protein expression in PVSMCs cultured in NG or HG.

Therefore, HG and AII may produce additive effects on 12-LO activation in VSMCs. Previous studies have shown that HG can enhance AII-induced hypertrophy in renal tubular cells (49).

12-LO products can be mitogenic and activate several systems linked to increased vascular disease and cell proliferation including protein kinase C (40, 50) and activity of the ras oncogene (51). Furthermore, 12-HETE can activate c-fos oncogene expression (52) and also lead to SMC migration (36). Inhibition of the LO pathway with baicalein markedly reduces excess proliferation of PVSMCs cultured in HG toward levels seen in NG and that LO inhibition can reduce AII-induced hypertrophic effects in VSMCs. This same concentration of baicalein inhibits 12-HETE formation in PVSMCs. Hence, excess VSMC growth under hyperglycemic conditions may be due to excess 12-LO activation. The marked activation of the 12-LO pathway by HG and AII in this model cell system suggests that 12-LO activation may be a key mechanism for accelerated vascular disease produced by hyperglycemia and AII in diabetes mellitus. Particularly so in view of recent preliminary evidence showing marked increases in 12-HETE excretion in patients with type II diabetes compared to controls (53).

C. hl 12-LO MEDIATION OF AII INDUCED HYPERTROPHY

Angiotensin II (AII) has major effects on vascular smooth muscle cell (SMC) growth in vitro and in vivo (38, 39, 54, 55). In particular, it has become clear that AII-induced SMC hypertrophy is accompanied by increases in the synthesis of key extracellular matrix proteins such as collagen and fibronectin (56).

It has previously been shown that the 12-lipoxygenase (12-LO) pathway of arachidonate metabolism plays an important role in AII action on growth and steroidogenesis in the adrenal (14, 40). Parts A and B demonstrate that a leukocyte type of 12-LO enzyme is present in porcine and human SMC (PSMC) and that AII can increase the formation of the 12-LO product, 12-hydroxyeicosatetraenoic acid (12-HETE) and also increase the expression of the 12-LO mRNA and protein (57). It has also been demonstrated that PSMC cultured in elevated glucose show increased activity and expression of 12-LO (57). However, the effects of high glucose (HG) conditions on AII-induced SMC hypertrophic responses have not previously been studied.

This part C elucidates the role of this 12-LO pathway on AII-induced SMC hypertrophic responses. Specifically, the role of the 12-LO pathway in AII-induced protein and fibronectin synthesis was determined. These responses were compared in PSMC cultured in normal glucose (NG) and HG. The results indicate that 12-LO activation plays a key role in AII-induced SMC hypertrophy and that AII effects are enhanced in PSMC cultured under HG conditions.

1. Description of Figures 21 to 25

Figure 21 depicts the dose-dependent effects of AII on total cell protein content in PSMC cultured in NG or HG. Results are expressed as mean ± SE from 4-6 experiments performed in triplicate. *, $p<0.03$ vs basal; **, $p<0.01$ vs basal; †, $p<0.05$ vs NG. Basal protein content, NG; 201±8 $\mu g/10^6$ cells and HG: 240±7 $\mu g/10^6$ cells.

Figure 22 depicts the effect of AT_1 (Losartan or DuP753) and AT_2 (PD123177) receptor antagonists on AII-induced increases in cellular protein content in

PSMC. Results are mean ± SE from three experiments performed in duplicate or triplicate. *, p<0.01 vs AII.

Figure 23 depicts the effect of a specific LO inhibitor (baicalein), and a CO inhibitor (ibuprofen) on AII-induced protein synthesis. Results are expressed as mean ± SE from three experiments performed in triplicate. *, p<0.01 vs basal; **, p<0.03 vs AII.

Figure 24 depicts the effects of HETEs on total cell protein content in NG (A) or HG (B). Results are mean ± SE from three to five experiments performed in triplicate. NG, A:*, p<0.02 vs basal; HG, B: *, p<0.01 vs basal; **, p<0.05 vs basal.

Figure 25 depicts the effect of AII and 12-HETE treatment for 40 hours on the levels of released fibronectin (A) and cell-associated fibronectin (B) in PSMC. Results are mean ± SE from four to five experiments performed in triplicate. A, released FN: *,**,***: p<0.001, <0.01, <0.05 vs basal resp.; †, p<0.03 vs. AII 10^{-7}M, †† p<0.02 vs. 12-HETE 10^{-7}M. B, cell-associated FN: *,**: p<0.001, <0.01 vs basal resp. †, p<0.01 vs. AII 10^{-7}M. In addition, AII and 12-HETE effects were significantly greater in HG (p<0.01). Basal FN values: Released, 12.1±0.7 $\mu g/10^6$ cells NG and 18.6±1.1 μg HG. Cell-associated, 2.0±0.3 $\mu g/10^6$ cells NG and 3.05±0.4 μg HG.

2. Materials and Methods

Culture of porcine aortic smooth muscle cells (PSMC): Primary cultures of PSMC were obtained as described earlier (28). Cells were maintained n Dulbecco's modified Eagle's medium (DME) containing NG (5.5 mM), and 10% fetal calf serum (FCS). For studies under hyperglycemic conditions, the cells were allowed to grow through at least 2 passages in

DME HG (25 mM) before use. Control for osmolalility were cells grown for at least two passages in NG+19.5 mM Mannose.

Measurement of total cellular protein content: PSMC in NG or HG (about 90% confluent) in 12-well plates were serum depleted for 24 hours by placing in medium containing 0.2% BSA and 0.4% FCS. This medium was then freshly added along with agonists such as AII (Peninsula Labs, Belmont, CA), 12-HETE or 15-HETE (Biomol, Plymouth Meeting, PA) and cells incubated for 40 hours at 37°C. HETEs were added from 1000-fold concentrates in dimethylsulfoxide (DMSO). The vehicle, DMSO, was added to the controls. In some experiments, prior to AII addition, cells were preincubated for 15 minutes with AII-receptor antagonists losartan (ATI) or PD123177 (AT2) (generous gifts from DuPont Merck, Wilmington, DE), or a specific LO inhibitor baicalein (Biomol) or a cyclooxygenase (CO) inhibitor, ibuprofen (Sigma). At the end of the incubation period, washed cell monolayers were lysed in 300 µl of 0.3N NaOH and protein content determined by Lowry's method.

Measurement of fibronectin (FN). Quiescent 80-90% confluent PSMC in NG or HG in 12 well dishes were placed in media containing 0.2% BSA and 0.4% FCS without phenol red and treated for 40 hours with agonists. The supernatants were assayed for released FN while washed cell layers were sonicated in 500 µl of 1% nonidet P-40 PBS and assayed for cell-associated FN. FN levels in all samples were determined by a double antibody sandwich ELISA assay using the methods provided by the manufacturer (DAKO Corporation, Carpinteria, CA) and Wolthuis et al. (59). A polyclonal rabbit anti-human FN (1:1000) was used as the coating antibody and the detection

antibody was a peroxidase-conjugated rabbit anti-human FN (1:2000).

Statistical Analysis: Results were analyzed using the Student's t-test. For multiple comparisons or dose responses, analysis of variance was utilized. Duncan's test was also used for multiple comparisons.

3. Results

Hypertrophic effects of AII in PSMC cultured in NG or HG: AII produced a dose-dependent increase in total cellular protein content in cells cultured in either NG or HG conditions (Figure 21). Furthermore, the effects of AII especially at 10^{-7} and 10^{-6} concentrations, were clearly potentiated by culturing the PSMC in HG for at least 2 passages (Figure 21). AII did not cause any significant increase in cell number in these experiments (results not shown).

In order to determine the AII receptor subtype involved in the changes in protein levels, additional experiments were performed using the selective AT1 receptor antagonist, losartan (DuP753) and the AT2 blocker, PD123177. As shown in Figure 22, DuP753, but not PD123177 blocked AII-induced increases in total cellular protein content. Neither the AT1 nor AT2 antagonist alone had any effect on basal protein content in either NG or HG (data not shown).

Effect of LO and CO inhibition on AII-induced increase in cellular protein content: Figure 23 demonstrates the effect of the specific LO inhibitor baicalein and specific CO inhibitor ibuprofen on AII-induced cellular protein content. Neither inhibitor alone significantly altered basal cellular protein levels. However, baicalein the LO inhibitor, completely blocked AII-induced protein increases. In

marked contrast, the CO inhibitor, ibuprofen, had no effect on AII action (Figure 23).

Direct effect of 12 and 15-LO products on cellular protein content: Part B shows that AII directly increases 12 and 15-HETE levels in PSMC cultured in HG and HG (57). The direct effect of addition of these LO products on cellular protein levels in PSMC cultured in NG and HG was therefore evaluated. As shown in Figure 24A, in NG, 12-HETE at 10^{-6} and 10^{-7} had similar stimulatory effects as AII (10^{-7}M) on total cell protein levels. However, the 15-LO product 15-HETE at 10^{-6}-10^{-8}M concentration had no effect on protein content (Figure 24A). Figure 24B shows the dose-dependent effects of the LO products on cellular protein content in PSMC cultured in HG. As in the NG condition, 12-HETE at 10^{-6} and 10^{-7}M showed similar stimulatory responses as AII. The effects of AII and also 12-HETE were enhanced in HG, with 12-HETE at 10^{-8}M now showing a significant response (Figure 24B). The effects of 15-HETE were also potentiated by HG culture conditions, with concentrations of 15-HETE at 10^{-6} to 10^{-8} now showing stimulatory effects. However, the effects of 15-HETE even at 10^{-6}M were less than the responses to AII (Figure 24B). Neither 12- nor 15-HETE changed the cell number.

Effect of AII and 12-HETE on fibronectin levels: Figure 25A compares the dose-related effects of AII and 12-HETE on released FN levels in PSMC cultured in NG or HG conditions. AII produced only a slight non-significant increase in released FN levels in PSMC cultured in NG. In contrast, AII, at all concentrations tested, stimulated released FN levels in cells cultured in HG. The maximal effect of AII was at 10^{-7}M concentration (Figure 25A). In NG,

12-HETE stimulated FN only at 10^{-7}M (Figure 25A). However, in HG, 12-HETE produced a greater stimulation with a dose-dependent effect from 10^{-7} to 10^{-9}M concentrations.

The changes in cell associated FN levels are shown in Figure 25B. Neither AII nor 12-HETE at 10^{-7} to 10^{-9}M significantly changed basal cell-associated FN levels in NG. In contrast, both AII and 12-HETE led to a dose-dependent stimulation of cellular FN levels in cells cultured in HG (Figure 25B).

Figure 26 is a bar graph which depicts the effect of AII on protein synthesis in PVSMC cultures in normal or high glucose.

Figure 27 depicts the effect of baicalein (10^{-6}M) a 12-LO inhibitor on smooth muscle cell growth in normal glucose (5.5 mM) and high glucose (25 mM) conditions. Cell number is significantly reduced by baicalein in high glucose only.

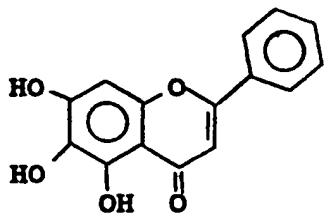
- ▽ High glucose without baicalein
- ▼ High glucose with baicalein
- o Normal glucose without baicalein
- ● Normal glucose with baicalein

D. Inhibition of 12-LO

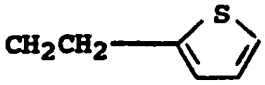
The activity of h1 12-LO is inhibited in vitro chemically by various compounds and by ribozyme cleavage of the corresponding mRNA.

1. Chemical Inhibition of h1 12-LO

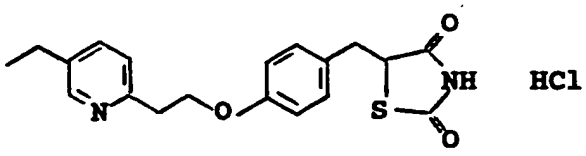
The h1 12-LO pathway is inhibited by various compounds, including baicalein



"compound 24"



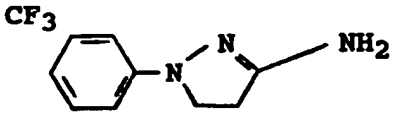
pioglitazone,



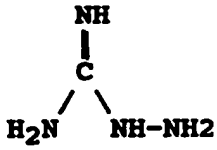
U-72, 107A

Empirical Formula:
$C_{19}H_{21}ClN_2O_3S$

BW755c,



aminoquanidine,



angiopeptin, BIM 23014 (D-Nal-Cys-Tyn-D-Trp-Lys-Val-Cys-Thr-NH_2); (See Circulation 88:11-14 (1993)); compound 10 (Boehringer Mannheim); (See Biochemical Society Transactions 21:661 (1993) and Patent WO 9216503); MDL 29311 (Merrell-Dow); (See Diabetes 42:1179-86 (1993)); 2-phenylmethyl-1-napthol; carvedilol, (1-[carbozoyly-(4)- oxy]-3-[Z methoxyphenoxyethyl amino] propanol-(2)), and antioxidants.

1,2 diacylglycerol blockers such as CT-1501R, CT-15436 and CT-1541 (products of Cell Therapeutics, Inc.) are of particular interest. 1,2 diacylglycerols provide key fatty acid substrates which are metabolized by the 12-LO enzyme to active products such as 12-HETE. Hence, DAG blockers are expected to reduce the formation of 12-LO products. One aspect of this invention, therefore, includes the use of DAG blockers to mediate the hl 12-LO pathway. Further, it is known that phosphatidic acid is converted to 1,2 diacylglycerols by phosphatidate phosphohydrolase (PAPh). Thus another aspect of the invention includes the use of inhibitors of that enzyme to mediate the hl 12-LO pathway.

EXAMPLE I

Baicalein is a specific inhibitor of the 12-lipoxygenase enzyme. This compound is obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Baicalein is added from a 1000-fold stock solution in dimethyl sulfoxide (DMSO) (2.7 mg in 1.0 ml DMSO, 10^{-2}M). This solution is prepared fresh and kept protected from light. The vehicle (0.1% DMSO) is added to the controls to control for solvent effects.

Baicalein can inhibit the leukocyte-type 12-LO and therefore inhibit angiotensin II-induced 12-HETE formation in cultured porcine aortic smooth muscle cells (PSMC).

Primary cultures of PSMC are obtained from smooth muscle sections of porcine aortas. Aortas obtained from a local abattoir are rinsed with DME media containing penicillin (100 U/ml) and streptomycin (100 μg/ml). Small pieces of the smooth muscle layer are placed on petri dishes, covered with media (DME + 15% FCS) and incubated at 37°C in a humidified

atmosphere. The smooth muscle cells that grow out of these explants are used for experiments up to passage 6. Cell are passaged using trypsin-EDTA.

About 24 hours prior to an experiment, confluent cells in 100 mm dishes are made quiescent by placing them in DME media containing 0.2% BSA and 0.4% FCS. THe cells are then washed twice with PBS, placed in DME containing 0.2% BSA (7.5 ml) and preincubated for 30 minutes with the addition of baicalein in the last 15 minutes. All (10^{-7}M) is then added for 10 minutes. The incubations are terminated by cooling the plates on ice. The supernates are removed and ethanol (1.35 ml/sample) added and saved at -70°C.

12-HETE Is extracted from the samples as follows: The pH of the cell supernates, which are now in 15% ethanol is adjusted to 3.5 with 0.1N HCl. Tritiated 12-HETE (650 cpm/sample) is added as recovery counts. Samples are loaded on Bond-Elut mini columns (Varian) which were prewashed with 2 ml methanol and 1 ml water. The 12-HETE is eluted from the column with 2 ml of ethyl acetate. This eluate is dried down under a stream of nitrogen gas in subdued light. The residue is reconstituted in 1 ml ethanol. 0.1 ml of this is counted to obtain recovery counts to calculate the percent recovery.

12-HETE in these extracts is quantitated by a specific radioimmunoassay as follows. 0.1 ml aliquots of ethanol extracts of 12-HETE are dried down under nitrogen gas in polypropylene tubes. A standard curve is set up made using authentic 12-HETE (BIOMOL) ranging from 10 pg/ml to 700 pg/ml. To each tube is then added 0.1 ml 12-HETE antibody (Advanced Magnetic, Boston), 0.1 ml tritiated 12-HETE (6000 cpm) and 0.1 ml of assay buffer. This mixture is incubated for 2 hours at room temperature in the

dark. The separation of bound from free 12-HETE is achieved by the addition of 0.7 ml activated charcoal solution and rapidly centrifuging. The radioactivity in the supernatants is determined by liquid scintillation counting. Concentrations of unknown samples are obtained from the standard curve plot of concentration vs. % bound.

Result

Treatment	Released 12-HETE pg per 10^6 cells
Basal	120 ± 10
AII (100 nM)	190 ± 28*
AII + baicalein (10 μM)	141 ± 26**
Baicalein (10 μM)	129 ± 12

EXAMPLE II

The effect of pioglitazone on the levels of 12-HETE in adrenal glomerulosa cells rats fed a high fructose diet:

Three sets of rats were used in this study which was done in collaboration with scientists at the University of Southern California Medical Center. One set received normal control diet, the second set received a diet high in fructose, while the third received the high fructose diet along with 40 mg/Kg pioglitazone orally. Animals were sacrificed about 6 weeks after the diet. The adrenal glands were removed and incubated in DME/F12 containing 0.5% BSA, 2.4 mg/ml collagenase and 0.15 mg/ml deoxyribonuclease (1.5 ml/6 glands) at 37°C for 1 hour with shaking and in an atmosphere of 95% oxygen + 5% carbon dioxide. After dilution with media containing 0.4% BSA, cells were dissociated from the tissue by aspiration. Cells were washed three times by centrifugation at 900 rpm and finally suspended in DME/F12 + 0.5% BSA to yield 100,000 cells/ml. Cell

suspensions were similarly prepared from the three groups of animals.

The above cell suspensions (1.5 ml) aliquots were incubated for 1 hour at 37°C with shaking. At the end of the incubation, the suspensions were saved at -70°C for the measurement of 12-HETE.

12-HETE in the incubates was extracted on Bond-Elut mini columns as described under Example I. Just prior to the extraction process, phenidone (10^{-6}M) was added to each sample to prevent on-specific LO product formation. Ethanol (final 15%) was then added to the samples followed by extraction.

12-HETE in the extracts was quantitated by radioimmunoassay as described under Example I.

Result:

Rat Adrenal Glomerulosa Cell
12-HETE levels (pg/ml)

Control Animals	Fructose Fed Animals	Fructose Fed + Pioglitazone
236 ± 6	618 ± 90	177 ± 18

EXAMPLE III

BW755c Inhibition of 12-LO

BW755c, an inhibitor of the LO pathway, was obtained as a gift from Burroughs Wellcome Laboratories, U.K. It was stored at 4°C. Stock solutions of 10^{-2}M BW755c were prepared by dissolving 2.6 mg in 1.0 ml water. This solution was prepared fresh, kept on ice and protected from light and air.

Experiments were performed with freshly isolated human adrenal glomerulosa cells to examine the effects of BW755c on 12-HETE formation. Human adrenal glomerulosa cells were prepared from fresh specimens of normal human adrenal tissues obtained

from surgery for renal or testicular carcinomas. The glomerulosa section was finely minced asceptically and incubated with collagenase (2.5 mg/ml) and DNase (0.15 mg/ml) in DME/F12 medium containing 0.2% BSA at 37°C. The cells are mechanically separated by gentle pipetting, filtered, centrifuged, washed twice and finally suspended in DME/F12 + 0.5% BSA to yield 100,000 cells/ml. BW755c (10^{-6}M-10^{-4}M) was then added and the cells (1.5 ml) preincubated for 15 minutes. Angiotensin II (10^{-8}M-10^{-10}M) was then added to the cells and the incubation carried on for 1.5 hours at 37°C. At the end of the incubation, a 200 µl aliquot of the cell suspension was removed for the measurement of aldosterone while the remaining 1.3 ml was rapidly put away at -70°C for the quantitation of 12-HETE. 1.3 ml of media alone was also saved as a blank.

12-HETE in the incubates was extracted and quantitated as described under Example I.

Result:

The Effects of BW755c on Basal and AII-Induced 12-HETE Production in Normal Adrenal Glomerulosa Cells

Treatment	12-HETE (pg/10^5 cells.h)	P
Basal	1739 ± 116	
AII (10^{-9} mol/L)	2626 ± 109	<0.01 vs. basal
AII + BW755c (10^{-6} mol/L)	2059 ± 421	>0.1 vs. AII
AII + BW755c (10^{-5} mol/L)	1780 ± 106	>0.01 vs. AII
BW755c (10_{-6} mol/L)	1847 ± 222	>0.6 vs. basal
BW755c (10^{-5} mol/L)	1928 ± 96	>0.3 vs. basal

The results clearly show that BW755c is a very effective dose dependent inhibitor of the formation of the 12-LO product 12-HETE in human adrenal glomerulosa cells.

EXAMPLE IV

Aminoguanidine, which is an inhibitor of nitric oxide synthase, has recently been shown by other workers to be a potential agent to prevent diabetic vascular dysfunction. Whether some of the effects of aminoguanidine (AG) may be via blockade of 12-HETE formation were examined by studying its effect on basal and angiotensin II-induced 12-HETE formation in PSMC.

Experiments to measure 12-HETE were performed with PSMC in a manner similar to that described under Example 1.

Results:

	12-HETE (pg/ml)
Control	18.8 ± 0.7
AII 10^{-7}M	28.6 ± 2.0
AII + AG (1.0 mM)	12.2 ± 1.3
AG (1.0 mM)	12.4 ± 2.2

Thus the preliminary results indicate that some of the effects of AG may be via inhibition of the 12-LO pathway by AG attenuation of basal and complete blocking of AII-induced 12-HETE formation.

4. Summary

Clear evidence indicates that AII produces hypertrophic responses in cultured aortic medial SMC (38, 54). The present study suggests that AII-induced increases in protein synthesis in PSMC are mediated predominately by the type 1 receptor since losartan but not PD123177 prevent AII effects. These results are similar to those in a recent report in rat SMC (60). The signal transduction mechanisms for AII-induced hypertrophic responses are not completely understood. AII can activate a phosphatidylinositol specific phospholipase C (PLC) leading to inositol triphosphate which can mobilize

calcium (61, 62). However, AII-induced activation of PLC and other phospholipases including PLD can generate diacylglycerols (DAG) (61, 63). These and other signals can then produce expression of certain oncogenes and growth factors (64-66).

It is known that activation of a 12-LO enzyme plays a key role in mediating both AII-induced steroidogenic and mitogenic responses in the adrenal cortex (14,40). Furthermore, AII increases both the activity and expression of a leukocyte type of 12-LO enzyme in PSMC (57). In this part C it is shown for the first time that the 12-LO pathway is particularly involved in AII-induced hypertrophy in PSMC. Support for this are the results showing that a relatively selective 12-LO inhibitor but not a CO inhibitor completely blocks AII-responses. Furthermore, a 12-LO product 12-HETE directly increases protein content to a greater extent than 15-HETE. The precise mechanism by which 12-HETE increases protein concentration was not assessed in the current report. However, previous studies have shown that 12-LO products can increase PKC activity and oncogene expression (40, 50, 52). In these studies, both AII and 12-HETE had only hypertrophic effects and no evidence of hyperplasia was seen.

Another major finding is the demonstration that HG culture potentiates the stimulatory effects of both AII and 12-HETE on protein synthesis in PSMC. A previous report has also revealed that HG can enhance AII-induced hypertrophic responses in murine proximal tubular cells (49). The effects of glucose on increasing protein synthesis in PSMC are not due simply to hyperosmolar effects as has been shown that similar concentrations of mannitol do not alter HG-induced increased growth of PSMC (28). The

precise mechanism of how glucose potentiates AII-growth responses will require further study. However, it has been reported that elevated glucose can lead to de-novo synthesis of DAG and activate PKC in vascular cells (67, 68). Furthermore, HG can increase the activity and expression of the 12-LO pathway (57). Therefore, increases in 12-HETE with subsequent increases in specific isoforms of PKC may be involved in upregulation of these responses. It is recognized, however, that many additional mechanisms are possible including changes in G proteins, receptor binding or changes in calcium mobilization.

In addition to changes in total protein, Part C indicates that both AII and 12-HETE can produce dose-dependent increases in cell associated and released FN levels only in PSMC cultured in HG. The effect of 12-HETE on FN levels could be seen at concentrations as low as 10^{-9}M. HG alone also produced increases in cell associated and released FN levels (data not shown). Additional studies will be needed to confirm whether the increases in fibronectin are due to changes in synthesis or reduced degradation. However, most reports have shown that glucose or AII induced increases in matrix proteins are primarily produced by increases in synthesis (69, 70).

Diabetes mellitus is associated with a substantially increased prevalence of hypertensive and atherosclerotic cardiovascular disease (58). Prolonged hyperglycemia induced biochemical changes have been associated with diabetes specific vascular disease (27). Enhanced responses to the vasopressor action of AII have also been shown in diabetics (37). The data set forth herein indicates that AII-induced

vascular hypertrophic responses may be accentuated in diabetes by activation of a 12-LO pathway in the vascular smooth muscle. New data also indicates that AII, by acting on the cardiac myocyte or fibroblast, may play a key role in cardiac hypertrophy and heart failure (39). Therefore, the 12-LO pathway may also mediate these cardiac actions of AII.

PDGF (BB form) has been clearly linked to atherosclerosis and cellular growth (39). PDGFBB was added to PVSMC resulting in significant increases in 12-HETE formation. Furthermore, PDGF increases 12-LO protein expression in PVSMC and in human fetal aortic SMC. For this experiment, cells were serum depleted for 24 hours and put in serum free media with either PDGF 10^{-10} or 10^{-11}M or TGFB 10^{-10} or 10^{-12}M. Proteins were isolated at 24 or 48 hours and Western immunoblot performed using the h1 12-LO peptide antibody. At 24 hours, PDGF 10^{-10} and 10^{-11}M produced a close-dependent increase in 12-LO expression. The PDGF effect was less pronounced at 48 hours. However, TGFB at 48 hours increased 12-LO expression. These results suggest that activation of the h1 12-LO may also mediate the vascular and renal actions of PDGF and TGFB (40).

2. Ribozyme Inhibition of 12-LO

The newly discovered h1 12-LO may be inhibited by ribozyme cleavage of the corresponding mRNA at any ribozyme cleavage site. Hammerhead ribozymes as described, for example, in Haseloff, et al., Nature 334:585-591 (1988) may be employed.

Chimeric ribozymes involving a combination of RNA and DNA sequences as disclosed for example in U.S. Patent 5,149,796 and 5,144,019 are useful h1 12-LO inhibitors. Modified ribozymes of increased stability in cell culture are preferred. See

Heidenreich, et al., J. Biol. Chem. 267:1904-1909 (1992), Abstract entitled "2'-Modified Hammerhead Ribozymes - Activity and Stability", Third International Symposium on Catalytic RNAs, Dec. 6-11, 1992, p. 28, and "RNA/DNA Chimeric Hammerhead-type Ribozymes Possessing Surprisingly High Cleavage Activity and Substrate Specificity", ibid., p. 35.

Description of Figures 28 to 34

Figure 28 depicts a chimeric ribozyme effective to cleave pl 12-LO RNA.

Figure 29 demonstrates cleavage of 12-LO RNA by the ribozyme of Figure 28.

Figure 30 is an autoradiogram illustrating that the desired cleavage occurred.

Figure 31 illustrates the efficiency of the cleavage reaction.

Figure 32 illustrates stability of the ribozyme of Figure 29.

Figure 33 illustrates important stability of the Figure 29 ribozyme in the presence of transfectum.

Figure 34 illustrates the efficiency of the ribozyme in cleaving porcine leucoyte, 12-LO and human 15-LO mRNA.

EXAMPLE OF RIBOZYME CLEAVAGE

This Example illustrates the cleavage of porcine 12-LO with a chimeric ribozyme as depicted by Figure 28.

Figure 29 indicates the effectiveness of the chimeric ribozyme. Lane 1 represents one control showing lack of cleavage of the 12-LO RNA 5' end in the presence of RNA and ribozyme in the absence of magnesium, a necessary co-factor for the reaction. Lane 2 represents a second control showing no cleavage in the absence of the ribozyme. Lanes 3-11

show efficient cleavage of the 5' end of the 12-LO RNA with the ribozyme. Separate reactions with varied concentrations of ribozyme and magnesium are illustrated.

Experimental Procedures

Materials: hl 12-LO RNA. Plasmid Neo pcDNA was obtained from Dr. Jai-Li Gu (this plasmid contained pl 12-LO cDNA). SP6, T7, and T3 RNA polymerases were obtained from BRL, Promega, and Strategen respectively. Calf Intestinal Phosphatase from United States Biochemicals. E.coR1, Bam Hi, HindIII, Afl II, and Kpn I were obtained from BRL and New England Biolabs [gamma 32p] ATP (6000 Ci/mmole), [alpha -32p] UTP (3000 Ci/mmole) were purchased from Dupont.

Transcription of RNA

The transcription of RNA from plasmid was accomplished using the procedure outlined by Promega. The procedure for making unlabelled and labelled RNA is given below.

High Specific Activity
RNA Probe Synthesis (20 ul)

4.0 ul	5x transcription buffer
2.0 ul	100 mM DTT
0.8 ul	RNasin ribonuclease inhibitor (25 units/ul)
4.0 ul	2.5 mM each of ATP, GTP, CTP and water (made by mixing together equal amounts of the rNTP Stocks)
2.4 ul	100 uM UTP (Final Con = 12 uM)
1.0 ul	Linearized plasmid DNA (~1.0 ug)
5.0 ul	100 mCi/ml [^{32}P] YTO
10-20	Units of SP6 RNA Polymerase

Add DEPC-treated water to a final volume of 20 ul.
Incubation at 37°C for 60 minutes.

5x RNA polymerase reaction buffer
.2 Tris-HCl (pH 8.0)
40 mM MgCl2
10 mM spermidine-HCl3
125 mM NaCl

Synthesis of Large
Amounts of Unlabelled RNA (200 ul)

40.0 ul	5x transcription buffer
20.0 ul	100 mM DTT
5.0 ul	RNasein
40.0 ul	2.5 mM each of ATP, GTP, cTP & UTP (made by mixing together equal amounts of the rNTP stocks)
2.0 ug	linearized plasmid DNA

100 units of RNA Polymerase and then add DEPC-treated water to a final volume of 200 ul.

Incubation at 37°C for 120 minutes.

Unlabelled RNA was further purified after adding 200 ul water to the sample using a QIAGEN procedure.

Adjust to the binding conditions by adding

[16 ul] 5 M NaCl
[10 ul] 1 M MOPS

The final salt concentration is approximately 250 mM.

Adjust the pipette to 600 ul and equilibrate a QIAGEN [tip-20] by pipetting [300 ul] of buffer A in and out.

Buffer A: 400 mM NaCl
50 mM MOPS
15% Ethanol
pH 7.00

Absorb the sample on the QIAGEN [tip-20] by pipetting it in and out four times.

Wash the QIAGEN tip-20 with buffer A as described (see general handling) to remove proteins, nucleotides and other impurities. If radioactive

triphosphates must be removed, a syringe should be used. Force 6.0 ml of buffer A through the column within two minutes.

Mix equal volumes of buffer C and E and elute the RNA with 600 ul this mixture.

Buffer C:	Buffer E:
1000 mM NaCl	1100 mM NaCl
50 mM MOPS	50 mM MOPS
15% ethanol	15% ethanol
pH 8.0	4 M Urea

Precipitate eluted RNA by adding 0.8 vol. Isopropanol. Incubate on ice for 20 minutes and spin at maximum speed in an eppendorf centrifuge for 30 minutes. WAsh the pellet carefully with 100% ethanol.

5′ - 32p Labeling of RNA

Unlabelled RNA is incubated with 2 units of calf intestinal alkaline phosphatase in 1 x Cipase buffer 50 mM MgCl, 0.1 mM ZnCl, 10 mM sperimidine 20 ul of total volume, at 37°C for 30 minutes after which an additional unit of enzyme was added and incubated for 15 minutes more. The reaction was then heated to 65°C for 10 minutes (to inactivate cipase enzyme) chilled on ice and the following reagents were added to the reaction.

Volume (ul)	Stock Solutions	Final Con
1	40 u/ul RNasin	1 u/ul
5	100 mM DTT	10 mM
2	10 uCi/ul [gamma-32p] ATP	10 mM
2	T4 polynucleotide kinase	0.4 u/ul
15	H20	
5	10x Kinas buffer 50 mM tris-HCl pH 7.6 10 mM MgCl2	

0.1 mM spermidine
0.1 mM EDTA

The reaction was incubated at 37°C for 90 minutes. Following transcription and 5'-end labelling, 1/2 volume of loading buffer (80% formamide, 0.05% Bromophenol blue and Xylene cyanol) was added and analyzed on 20% denaturing gel. After exposure the bands excised and RNA was recovered by crush and soak method (0.6 M NH OAc pH 8.9, 0.1 mM EDTA and 0.1% SDS). RNA activity was determined by scintillation counting.

In Vitro Ribozyme Cleavage of 12-LO RNA

A 10 ul volume containing ~ 50 ng of ribozyme and 50,000 CPM of target RNA (either 5'-end or internal labelled RNA) in 50 mM Tris-HCl pH 8.0 was mixed with 1 ul of 200 mM MgCl2 to start the reaction and further incubated over night. All reactions were stopped by the addition of an 80% formamide. Loading buffer was added and the samples were heated to 85°C for one minute, chilled on ice and electrophoresed in a 10% polyacrylamide, 7M urea gel.

Results and Discussion

A hammerhead ribozyme (hybrid DNA-RNA oligonucleotides, Taylor et al 1992) was designed to cleave the GUC sequence of the 5'-end of porcine 12-LO mRNA substrate in vitro. The cleavage reaction can occur at physiological temperature and required only the presence of divalent cations.

As shown in Figure 28 the chimeric DNA-RNA hybrid ribozyme used in these studies consists of 42-nt, which contained only 12 ribonucleotides. This ribozyme also contains 3 phosphorothiorate groups at the 3'-terminal.

The runoff transcript RNA (12-LO) ~ 200 nt length, either generally labelled or 5'-end labelled RNAs transcribed from E.CoRl linearized plasmid were

purified by 10% PAGE. This substrate RNA and ribozyme (either 5'-end labelled or cold ribozyme) were used to determine the specificity of ribozyme cleavage and to optimize the reaction conditions in vitro.

Initially the cleavage reactions were performed with heating (see Narida et al. 1992) at 85°C for five minutes in a solution of 05 mM Tris-HCl pH 8.0 in separate tubes, either ribozyme (at different concentrations) in one tube and substrate RNA (100,000 CPM) in another. This was followed by cooling to room temperature, addition of 20 mM MgCl2 to each tube and further incubation at 55°C for 15 minutes. The cleavage reactions were initiated by mixing equal volume of the target RNA and ribozyme from the above two tubes for a typical final volume of 10 ul per reaction and further incubation at 37°C for overnight (lanes 1, 2, and 3 in Figure 29). in the same fashion, another batch of experiments (lanes 4, 5 and 6) target RNA and ribozyme were heated at 85°C and chilled on ice. The cleavage reactions were initiated with mixing equal volume of target and ribozyme and incubation continued at 37°C as described above. Further, the cleavage reactions seen in lanes 7, 8 and 9 were performed by mixing the target and ribozyme without heating either ribozyme or target RNA. All the above reactions were further incubated at 37°C for overnight.

Figure 29 shows the predicted cleavage products of these three batches of reactions obtained by different combinations of conditions and relative cleavage efficiencies (with denaturing without denaturing). The amount of full-length uncleaved 12-LO RNA target substrate decreased substantially in the presence of the ribozyme when compared to the

control: lanes 1 and 2 in Figure 29. All major cleavage products were observed at the predicted position. It was found that the strongest cleavage products were observed when the target RNA and ribozyme were mixed directly with MgCl2 with preheating to 85°C. Whereas when the target and ribozyme were incubated at 85°C prior to cleavage, decreased amounts of cleavage product, as well as increased degradation of both target RNA and ribozyme were noticed. These results confirm that the structure adopted by the target 12-LO RNA substrate at 37°C allows subsequent cleavage. Under these assay conditions the efficiency of cleavage was not significantly dependent on the denaturing of either ribozyme or substrate RNA.

To confirm that the observed cleavage product was derived form the respective 5′-end, the internally labelled transcript of 200 nt 12-LO RNA was treated with various concentrations of cold ribozyme as described above. The migration of the cleaved radiolabeled product was analyzed in comparison with 5′-32p cleavage product. The cleavage appears to have occurred at a site common to both transcripts as expected at the 5′-end. The migration of product was observed by autoradiogram (Figure 30).

The efficiency of ribozyme cleavage was then addressed. Figure 30 illustrates that the cleavage product was detectable at ribozyme concentrations as low as 10 ng; cleavage product formation was optimal at 25-50 ng of ribozyme, at the physiological temperature of 37°C. Increasing amounts of the cleavage product was also observed with increasing the time. Cleavage products were still detectable after 15 minutes incubation of target RNA with

ribozyme and complete cleavage can be observed within two hours (Figure 31).

Figure 31 illustrates that the RNA strand scission can occur at various temperatures, the extent of cleavage increasing with increasing temperature, but the positional specificity of the strand cleavage is optimal only at physiological temperature, i.e., 37°C-42°C diminished dramatically above that temperature. Even at 55°C and incubation for two hours, no cleavage product was noticed. This observation differs from earlier reports. Nerida et al, 1992 reported that the optimal temperature for the chimeric ribozyme is 55°C. The present data suggests that the 12-LO RNA structure essential for the cleavage may be unable to form at higher temperature.

Further, the stability of the ribozyme was addressed. Increasing the catalytic RNAs stability in vivo is an important aspect for enhancing catalytic function. Chimeric ribozyme containing phosphorothioate groups at only three 3'-terminal positions were used. When this ribozyme was incubated with cells in culture more than 80% degradation was observed within two minutes (Figure 32), where as when this ribozyme was incubated in the presence of transfectum (Promega) under similar conditions, it exhibited considerably greater stability (Figure 33).

Figure 34 illustrates the efficiency of the ribozyme in cleaving porcine leucoyte, 12-LO and human 15-LO mRNA. The first four lanes show that the expected 12-LO RNA fragments are attained only when Mg and ribozyme are added. Similarly, the expected 15-LO RNA fragments are seen when adding ribozyme and Mg to labelled 15-LO mRNA.

Effect of Ribozyme to 12-LO protein expression in cultured PVSMC. To assay ribozyme cleavage in vitro, the porcine 12-LO cDNA was inserted in the order from 5′ to 3′ downstream from the SP6 promoter of pcDNA1neo plasmid and (Invitrogen, CA) was linearized with EcoR1. The SP6 RNA polymerase promoter produces the sense transcript for making 12-LO mRNA. Transcription mixture contained 1 μg linearized DNA, 50 μci ^{32}P UTP and 10U SP6 RNA polymerase (Promega) and incubated at 40°C for one hour. The product was treated with 1U of RNase-free DNase (Promega) at 37°C for 15 minutes and extracted with phenol/chloroform/isoamyl alcohol, followed by chromatography on a Sephadex G-50 spin column (5 prime->3 prime, Inc., PA) and electrophoresis in a 5% polyacrylamide-8M urea gel. The gel was autoradiographed. A full size of 210 nucleotides of transcribed target mRNA band was trimmed from the gel and soaked in NES (0.5M NH_4OAC, 0.1M EDTA, 0.1% SDS) overnight. The eluted RNA solution was precipitated by ethanol and dissolved in 50 μl water. The ribozyme in vitro cleavage was performed in a 10 μl reaction volume containing 50 mM TrisCl, pH 8, 1 mM EDTA, 20 mM $Mgcl_2$, varying amount of ribozyme and 1 X 10^5 cpm target RNA incubation at 37°C for overnight. The products were analyzed in a 10% polyacrylamide-8M urea gel and autoradiographed.

Figure 35 shows the effect of the ribozyme on 12-LO protein expression in PVSMC. The ribozyme was introduced into PVSMC using a liposome technique. The cells are 60-80% confluent at transfection. Fox 2 X 10^5 cells, 20 μg transfection (Promega, CO) and ribozyme (4-12 μg) are added. Cells were left in this condition for 48-72 hours. Ribozyme was added again after the first 12 hours. Sense and antisense

oligo DNA molecules were added for similar time periods. Cells and 12-LO protein were prepared as previously described and 12-LO protein expression was determined using Western blot. The data in Figure 35 indicates almost 80% reduction of 12-LO protein at 48 hours and 50% at 72 hours. Sense DNA had no effect, however. Antisense DNA had no effect at 48 hours, but at 72 hours, did reduce 12-LO protein expression by 80%. However, in this experiment, four times as much antisense DNA was added as compared to ribozyme.

E. Therapeutic Uses/Implications of Pharmacologic or Molecular Inhibition of Human Leukocyte 12-Lipoxygenase

1. Inhibition of Angiotensin II-induced vascular, renal, cardiac and adrenal actions.

Vascular.

Vascular smooth muscle proliferation and/or hypertrophy are key components of atherosclerotic and hypertensive cardiovascular disease. The current application provides evidence that activation of a hl type 12-LO can mediate several of the growth promoting actions of AII. The use of inhibitors of hl 12-LO pathway activation should prove highly useful as antihypertensive agents and agents to reduce atherosclerotic vascular disease due to locally formed or circulating AII in both non-hypertensive and hypertensive patients. These agents will especially be useful to reduce vascular disease progression in patients with diabetes since hyperglycemia enhances both the activation of the 12-LO pathway and AII-induced vascular effects.

Renal.

AII also plays a prominent role in the regulation of renal blood flow, glomerular filtration rate and mesangial cell growth. Increasing evidence supports

the pathogenic role of circulating or locally formed AII on accelerated kidney disease in states such as diabetes. Converting enzyme inhibitors which reduce AII formation have clearly shown marked protective effects in renal disease progression in diabetics and subjects with hypertension. A recent article (41) confirms and extends the original observation that the reno-vascular actions of AII are mediated by activation of a 12-lipoxygenase pathway. Therefore, blockage of the 12-LO pathway would be expected to preserve kidney function in states of excess AII formation or action including diabetes or hypertension.

Cardiac.

AII is increasingly thought to play a prominent role in pathogenic left ventricular hypertrophy and cardiac dysfunction seen in congestive heart failure and hypertensive or diabetic cardiomyopathy. Preliminary evidence suggests that hl 12-LO is expressed in the human heart using in situ hybridization techniques identical to those shown for the adrenal. Therefore, it is possible that cardiac AII action may also involve activation of a hl type of 12-LO. Thus inhibition of hl 12-LO could be a new treatment to prevent left ventricular hypertrophy in hypertensive and diabetic states or in other conditions associated with increased local AII formation.

Adrenal.

It has been clearly demonstrated that AII-induced aldosterone formation involves activation of a hl type 12-LO. Furthermore, AII regulates expression of 12-LO in human adrenal cells. Therefore blockade of 12-LO activation would be a new way to reduce aldosterone formation in states such as congestive

heart failure, liver disease and certain forms of hypertensions in which excess adlosterone leads to salt-retention, volume overload and hyperkalemia and hypomagesemia.

2. Reduced Atherosclerosis (Primary) or Neointimal Proliferation Associated With Angioplasty or Cardiac Transplantation.

Local AII formation is one factor associated with accelerated atherosclerosis. However, factors such as growth factors PDGF, TGFB and cytosines such as interleukin 1 have been proposed to be involved in these processes. The application indicates that PDGF and TGFB can increase 12-LO activation or expression. Furthermore, it is already documented that interleukin 1 and TNF can increase 12-HETE levels in arterial SMC (42). Therefore, activation of a 12-LO pathway could be an important mediator of the cellular growth and inflammatory actions of these agents. Thus, inhibition of 12-LO activation could be useful in preventing primary atheroscerotic vascular disease or disease induced by manipulation including angioplasty or transplant. Indeed new evidence shows that 12-HETE (10^{-7}M) added for 30 minutes to human aortic endothelial cells in culture significantly increases monocyte binding (54±5 cells to 72±5 cells per high power field p 20.05 n=6). Since monocyte binding is a key early step in atherosclerotic and inflammatory reactions, it supports the potential utility of selective inhibition of 12-LO activation in vascular disease.

3. Inhibition of Vascular and Kidney Disease Associated With Diabetes.

Both type I and type II diabetes is associated with complications primarily described as microvascular (eye, renal) or macro vascular (blood

vessel, heart). The application describes the particular effects of hyperglycemia on activation of hl 12-LO activity and expression in VSMC. Furthermore, inhibition of glucose induced cellular proliferation matrix formation and enhancement of AII action is shown with use of inhibitors of 12-LO activity. Hyperglycemia is linked to accelerated micro and macro vascular disease. Furthermore, glucose is linked to accelerated diabetic complications by forming non-enzymatic glycation end products or proteins. The application describes evidence that aminoguanidine, which is reported to reduce diabetic complications by blocking the formation of these glycated products, also reduces 12-LO activation. Furthermore, a glycated-end product of albumin added to PVSMC increases 12-HETE (unpublished observation). Therefore, blockade of 12-LO could provide a new method to reduce the excess cell proliferation or matrix protein deposition associated with hyperglycemic complications. Prolonged hyperglycemia is also linked to decreases in insulin action ("insulin resistance") and to impaired insulin secretion. Therefore, blockade of hl 12-LO to maintain normal insulin action and improve insulin secretion in the presence of continued hyperglycemia is indicated.

Another important process associated with hyperglycemia and diabetes complications is reduced action of nitric oxide (NO). NO is now thought to be the most important endothelial desired relaxation factor. NO plays a key role in maintaining normal blood pressure and preventing accelerated atherosclerosis by reducing monocyte binding to the endothelium and vascular smooth muscle cell growth. Hyperglycemia by activation of free radical

inactivates NO. Since LO activation produces potent lipid peroxides and free radicals, it is certainly possible that increased 12-LO activation by glucose mediates this process. Therefore, blockade of 12-LO activation could provide a new treatment to maintain normal NO activity in the vasculature exposed to elevated glucose or other factors associated with increased LO activity (oxidized or glycated lipids or proteins).

4. Prevention of Pancreatic Islet Cell Damage in Autoimmune (Type 1) Diabetes or During Islet Cell Transplantation to Reverse Diabetes.

Evidence in rats shows that a 12-LO analogous to the h1 type of 12-LO is found exclusively in the beta (insulin producing cells) of the pancreas. A leucocyte type of 12-LO is found in porcine islets using RT-PCR. Finally, immunohistochemical analysis in human pancreas shows staining for h1 12-LO in human islets.

Current information suggests that cytosines such as IL, and TNF play a key role in autoimmune destruction of the beta cells in type I diabetes or in islet cell rejection. Inducible types of NO provided by cytokine activation plays an important destructive role in beta cell damage. A recent paper (43) has shown that two inhibitors of the LO pathway can markedly reduce inducible NO formation in macrophages treated with cytokines. Furthermore, a recent abstract presented to the European Association for the Study of Diabetes in 1993 (44) showed that these same LO inhibitors protected rat islets form damage caused by cytosine induction of nitric oxide.

Therefore, it is proposed that inhibition of the h1 type of 12-LO could prevent the development of type I diabetes in susceptible patients.

Furthermore, use of an LO inhibitor systemically or locally (in islets) may reduce or prevent rejection of islets used for transplantation to reverse established type I diabetes.

EXAMPLE V

This Example indicates that blockage of hl 12-LO is useful as a treatment for human breast cancer. Pursuant to the experiment, baicalein (10^{-3}M) added to the MCF-7 cells, an established human breast cancer cell line, markedly reduces cellular mitogenesis as compared to cells grown in serum + DMSO vehicle alone. The bar graph shown in Figure 36 indicates actual cell numbers in the two treatment groups at several time periods.

5. Prevention of Cancer Cell Growth and/or Metastases.

Evidence shows that a hl type of 12-LO is overexpressed in two breast cancer cell lines compared to normal breast epithelial cells derived from reduction mammoplasty. Previous studies suggest that 12-LO products can lead to cell growth and tumor cell adhesion. One aspect of this invention entails blockade of hl 12-LO to reduce the growth or metastatic potential of certain cancers characterized by increased activity or action of this 12-LO pathway.

6. Diagnostic Assays Utilizing hl 12-LO Immunogen.

Immunohistochemical analysis with peptide antibodies has shown that hl 12-LO is expressed in human pancreatic islets. It is also known that blockade of the 12-LO pathway can prevent islet cell damage. It appears that antibodies to hl 12-LO circulate in pre-diabetic patients at risk for developing Type I (autoimmune diabetes). In addition, hl 12-LO antibodies would be present in

patients undergoing immune rejection of transplanted pancreatic tissue or islets. Accordingly, another aspect of this invention includes assays, e.g., of the ELISA type, in which a physiological specimen from a human patient is utilized for screening of individuals at risk for Type I diabetes. In addition, such a test would be useful to diagnose rejection of islet cells or pancreatic tissue given to diabetic patients in an attempt to reverse the diabetic state. In such assays, the immunogen is is all or part of hl 12-LO, preferably isolated and purified for use in the assay.

The hl 12-LO utilized in such diagnostic assays may either be purified or an isolated naturally occurring enzyme or a synthetically produced polynucleotide. A preferred natural or synthethic polynucleotide includes SEQ ID NO: 15.

7. PDGF Enhancement of 12-LO Activity in VSMC.

Yet another therapeutic aspect of the invention implicates the discovery that PDGF increases 12-LO activity and expression in vascular smooth muscle. Hence, enhanced hl 12-LO activity and expression in response to PDGF is indicated as a key mechanism for PDGF-induced vascular migration or proliferation observed in atherosclerosis.

REFERENCES

1. Walstra, P., et al. Biochim Biophys Acta 921:312-319 (1987).

2. Takahashi, Y., et al. Arch Biochem Biophys 266:613-621 (1988).

3. Hamberg, M., et al. Proc. Natl. Acad. Sci. USA 71:3400-3404 (1974)

4. Nugteren, D.H., Biochim Biophys Acts 380:299-307 (1975).

5. Yoshimoto, T., et al., Biochem Biophys Acta 713:638-646 (1982).

6. Ueda, N., et al. J. Bio. Chem. 265:2311-2316 (1990).

7. Natarajan, R., et al., J. Clin. Endocrinol. Metabl. 67:584-591 (1988).

8. Nataragan, R., et al. Am. J. Hypertens 5:800-805 (1992).

9. Curnow, K.M., et al., Mol. Endocrionl 5:1513-1522 (1991).

10. Laemmli, U.K,. Nature 227:680-685 (1970).

11. Towbin, H., et al. Proc Natl. Acad. Sci. 76:4350-4354 (1979).

12. Maruyama, T., et al., J. Histochem. Cytochem. 37:1125-1131 (1989).

13. Yoshimoto, T., et al., Proc. Natl. Acad. Sci. USA 87:2142-2146 (1990).

14. Nadler, J.L., et al., J. Clin. Invest. 80:1763-1769 (1987).

15. Izumi, T., et al., Proc. Natl. Acad. Sci. USA 87:7477-7481 (1990).

16. Sigal, Et., et al. Biochem Biophys Res. Commun. 157:457-464 (1988).

17. Tso, J.Y., et al., Nucleic Acid Resarch 13:2485-2502 (1985).

18. Funk, C.D., et al. Proc. Natl. Acad. Sci. 87:5638-5642 (1990).

19. Bachmann, B., et al., Nucleic Acid Resarch 18:1309 (1990).

20. Sanger, F, Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977).

21. Innis, M.A., et al., Proc. Natl. Acad. Sci. USA 85:9436-9440 (1988).

22. Kunkel, L.M., Proc. Natl. Acad. Sci. USA 74:1245-1249 (1977).

23. Fourney, R.M., et al. Focus 10:1,5-6 (1988).

24. Toshiya, A., et al., J. Biol. Chem. 267:12188-12191 (1992).

25. Yoshimoto, T., et al., Biochem. Biophys. Res. Commun. 172:1230-1235 (1990).

26. Stern, N., et al., J. Am. J. Physiol. 257:H434-443 (1989).

27. Ruderman, N., et al. FASEB J 6:2905-2914 (1992).

28. Nataragan, et al. Biochem. Biophys. Res. Commun. 187:552-560 (1992).

29. Hajjar, D.P., et al., FASEB J 6:2933-2941 (1992).

30. Yla-Hertualla, S., et al., Proc. Natl. Acad. Sci. USA 87:6959-6963 (1990).

31. Parthasarathy, S., et al., Proc. Natl. Acad. Sci. USA 86:1046-1050 (1989).

32. Wang, T., et al., Biochim. Biophys. Acta 1084:129-138 (1991)

33. Setty, B.N.Y., et al. J. Clin. Invest. 77:202-211 (1986).

34. Brown, M.L., J. Clin. Invest. 82:2136-2141 (1988).

35. Setty, B.N.Y., et al., J. Biol. Chem. 262:17613-17622 (1987).

36. Nakao, J., et al., Atherosclerosis 44:339-342 (1982).

37. Weidman, P., et al. Hypertension 7:Suppl. II, II-33-II-42 (1985).

38. Geisterfer, A.A., et al, Circ. Res. 62:749-756 (1988).

39. Daemen, J.J.A.P., Circ. Res. 68:450-456 (1991).

40. Natarajan R., et al., Endocrinology 131:1174-1180 (1992).

41. Natarajan, R., et al., Mol. Cell. Endocrinol. 72:73-80 (1990).

42. Yokoyama, C., et al. J. Biol. Chem. 261:16714-16721 (1986).

43. Yoshimoto, T., et al., Biochem Biophys Res. Commun 172:1230-1235 (1990).

44. Hansbrough, J.R., et al., J. Biol. Chem. 265:1771-1776 (1990).

45. DeMarzo, N., et al., Am. J. Physiol. 262:L198-207 (1992).

46. Koibuchi, Y., et al., Clin. Res. 40:150 (abstr.) (1992)

47. Metz, S., et al. Proc. Natl. Acad. Sci. USA 82:198-202 (1985).

48. McClain, D.A., et al., Proc. Natl. Acad. Sci. USA 89:8150-8154 (1992).

49. Wolf, G., et al., Biochem. Biopys. Res. Commun. 176:902-909 (1991).

50. Shearman, M.S., FEBS Lett. 243:177-182 (1989).

51. Yu, C.L., et al. Mol. Cell. Biol. 10:6683-6689 (1990).

52. Haliday, E., et al., EMBO J. 10:109-115 (1991).

53. Jost-Vu, E., et al., Clin. Res. 40:106 (abstr.). (1992).

54. Berk, B.C., et al., Hypertension 13:305-314 (1989).

55. Powell, J.S., et al., Science 245:186-188 (1989).

56. Kato, H., et al., Hypertension 9:17-22 (1991).

57. Natarajan, R., et al., Proc. Natl. Acad. Sci. USA 90:4947-4951 (1993).

58. Colwell, J.A., et al., Am. J. Med. Suppl. 67-80 (1983).

59. Wolthuis, A., et al., Kidney Int. 41:124-131 (1992).

60. Millet, D., et al. Eur. J. Biochem. 206:367-372 (1992).

61. Nabika, T., et al. J. Biol. Chem. 260:4661-4670 (1985).

62. Alexander, R.W., et al., Hypertension 7:447-451 (1985).

63. Lassegue, B., et al., Biochem J. 276:19-25 (1991).

64. Taubman, M.B., et al. J. Biol. Chem. 264:526-530 (1989).

65. Itoh, H., et al., Biochem. Biophys Res Commun 176:1601-1609 (1991).

66. Naftilan, A., et al., J. Clin. Invest. 83:1419-1424 (1989).

67. Lee, T.S., et al., Proc. Natl. Acad. Sci. USA 86:5141-5145 (1989).

68. Tesfamariam, B., et al., J. Clin. Invest 87:1643-1648 (1991).

69. Ayo, S.H., et al., Am J. Physiol. 260:F185-F191 (1991).

70. Sharifi, B.G., et al., J. Biol. Chem. 267:23910-23915 (1992).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Jerry L. Nadler
Rama Devi Natarajan
Jiali Gu

(ii) TITLE OF INVENTION: Human Leukocyte 12-Lipoxygenase Mediation of its Pathway, and Consequences Thereof

(iii) NUMBER OF SEQUENCES: 16

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: City of Hope

(B) STREET: 1500 East Duarte Road

(C) CITY: Duarte

(D) STATE: California

(E) COUNTRY: United States of America

(F) ZIP: 91010-0269

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3M Double Density 5 1/4" diskette

(B) COMPUTER: Wang PC

(C) OPERATING SYSTEM: MS-DOS (R) Version 3.30

(D) SOFTWARE: Microsoft (R)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 4 January 1994

(C) CLASSIFICATION: Unknown

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 07/936,660

(B) FILING DATE: 28 August 1992

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Irons, Edward S.

(B) REGISTRATION NUMBER: 16,541

(C) REFERENCE/DOCKET NUMBER: None

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (202) 783-6040

(B) TELEFAX: (202) 783-6031

(C) TELEX: None

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AACTCAAGGT GGAAGTACCG GAG 23

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

ATATAGTTTG GCCCCAGCCA TATTC 25

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGGCTCAGGA CGCCGTTGCC C 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTCAGTGTAG ACGTGTCGGA G 21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

ACTGCTGGTT TGTGAAACTG CGC 23

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATGTATGCCG GTGCTGGCTA TATTTAG 27

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AATTAACCCA TCCTTCCAGT TAC 23

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

TCAGGATGCG GTCGCCCTCC AC 22

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCCATCACCA TCTTCCAGGA G 21

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GTTGTCATGG ATGACCTTGG C 21

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTAAGCAGTT GGTGGTGCAG G 21

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GATGATCTAC CTCCAAATAT G 21

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CTGGCCCCAG AAGATCTGAT C 21

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GTTTGAGGGC CATCTCCAGA GC 22

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 239

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AAACGGCACC TCCTTCAGGA TGACGCGTGG TTCTGCAATT 40
GGATCTCCGT GCAGGGTCCG GGAGCAAACG GGGACGAGTT 80
CAGGTTCCCC TGCTACCGCT GGGTGGAGGG CGACCGCATC 120
CTGAGCCTCC CTGAGGGCAC TGCCCGCACA GTGGTCGATG 160
ACCCTCAAGG CCTGTTCAAG AAACACAGGG AGGAGGAGCT 200
GGCAGAGAGA AGGAAGCTGT ATCGGTGGGG TAACTGGAA 239

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 239

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

AAACGGCACC TCCTTCAGGA TGACGCGTGG TTCTGCAATT 40
GGATCTCCGT GCAGGGCCCG GGAGCAAATG GGGACGAGTT 80
CAGGTTCCCC TGCTACCGCT GGGTGGAGGG CGACCGCATC 120
CTGAGCCTCC CTGAGGGCAC TGCCCGCACA GTGGTCGATG 160
ACCCTCAAGG CCTGTTCAAG AAACACAGGG AGGAGGAGCT 200
GGCAGAGAGA AGGAAGCTGT ATCGGTGGGG TAACTGGAA 239

CLAIMS:

1. A method for screening a diabetic patient at risk for development of Type I (autoimmune) diabetes which comprises:

(i) obtaining a physiological specimen from said patient;

(ii) determining whether antibodies to human leukoycte 12-lipoxygenase are present in said specimen;

wherein the presence of said antibodies is indicative that such patient is at risk of developing Type I (autoimmune) diabetes.

2. A method as defined by claim 1 in which said specimen is blood.

3. A method for diagnosing the immune rejection of pancreatic tissues or islets by patients in which said pancreatic tissues or islets have been transplanted which comprises:

(i) obtaining a physiological specimen from said patient;

(ii) determining whether antibodies to human leukoycte 12-lipoxygenase are present in said specimen;

wherein the presence of said antibodies is indicative that such patient is at risk of developing Type I (autoimmune) diabetes.

4. In an ELISA, the improvement which consists essentially of an antigen consisting of human leukocyte 12-lipoxygenase which reacts with a human leukocyte 12-lipoxygenase antibody.

5. An ELISA as defined by claim 4 in which said antigen includes SEQ ID NO: 15.

6. A method which comprises:

(i) obtaining a physiological specimen from a human;

(ii) determining whether antibodies to human leukocyte 12-lipoxygenase are present in said specimen.

7. A method for the treatment of human breast cancer which comprises administration to a human patient suffering therefrom a human leukocyte 12-lipoxygenase inhibitor in an amount effective to retard or inihbit the expression of said enzyme.

8. A method as defined by claim 7 in which said human 12-lipoxygenase inhibitor is biacalein, pioglitazone, aminoquanidine, or a ribozyme which leaves human 12-lipoxygenase mRNA.

9. A method which comprises:

(i) administering platelet derived growth factor to a mammal;

(ii) determining whether said platelet derived growth factor increase 12-lipoxygenase activity and expression in said mammal.

10. Isolated and purified 12-LO expressed by human vascular smooth muscle cells or human adrenal cells or mononuclear cells or endothelial cells, said isolated and purified 12-LO being distinct from that found in human platelets and distinct from human 15-LO.

11. A method for treating human vascular disease which comprises inhibiting the expression of the human 12-LO as defined by claim 10 to mediate angiotensin II and glucose induced vascular and renal actions.

12. A method as defined by claim 11 in which said mediation is accomplished by administration of a 12-LO inhibitor--e.g., baicalein.

13. Isolated and purified human 12-LO RNA from human vascular smooth muscle cells or adrenal cells or mononuclear cells or endothelial cells, said

isolated and purified 12-LO being distinct from that found in human platelets.

14. A method which comprises regulating the protein and RNA expression of the human 12-LO defined by claim 10 by culturing said cells in vitro in a medium in which the concentration of angiotensin II is controlled.

15. A method as defined by claim 14 in which the concentration of angiotensin II is increased to upregulate expression of 12-LO protein and RNA or decreased to down regulate expression of 12-LO protein and RNA.

16. A method for mediating angiotensin II and glucose induced vascular and renal action by controlled activation of the expression of 12-LO expressed by human vascular smooth muscle or adrenal or endothelial or mononuclear cells.

17. A method as defined by claim 16 in which said activation is controlled by regulating the level of ambient glucose concentration bathing said cells.

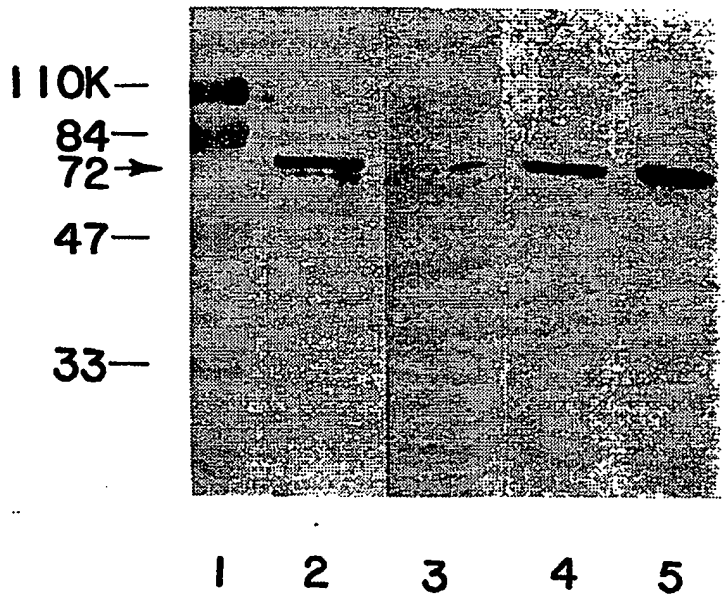


FIG. 1

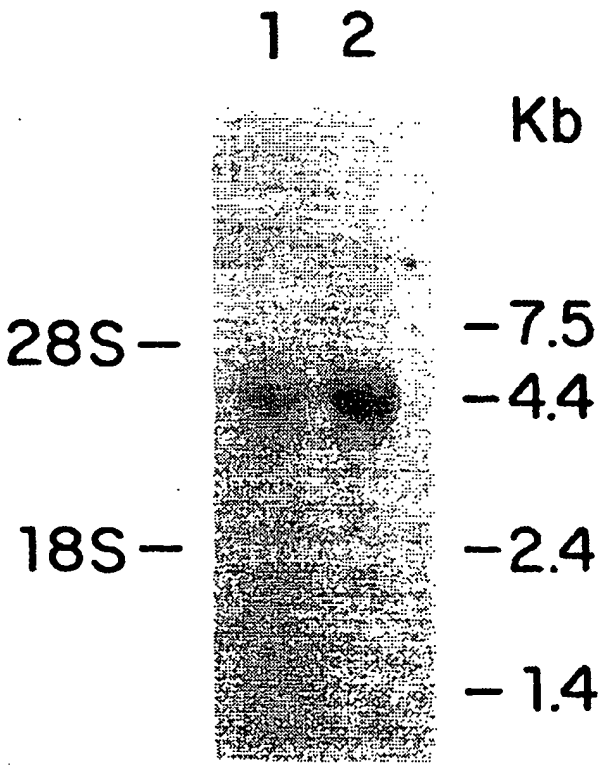


FIG. 2

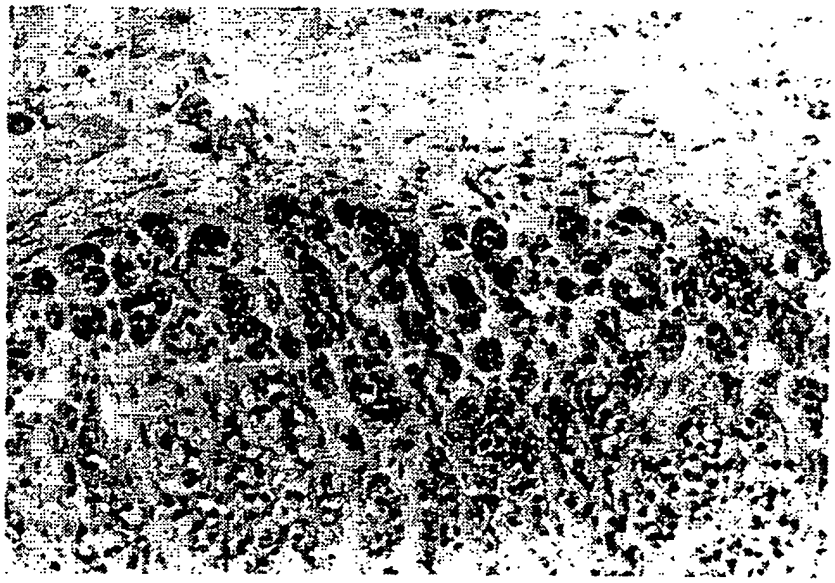


FIG. 3A

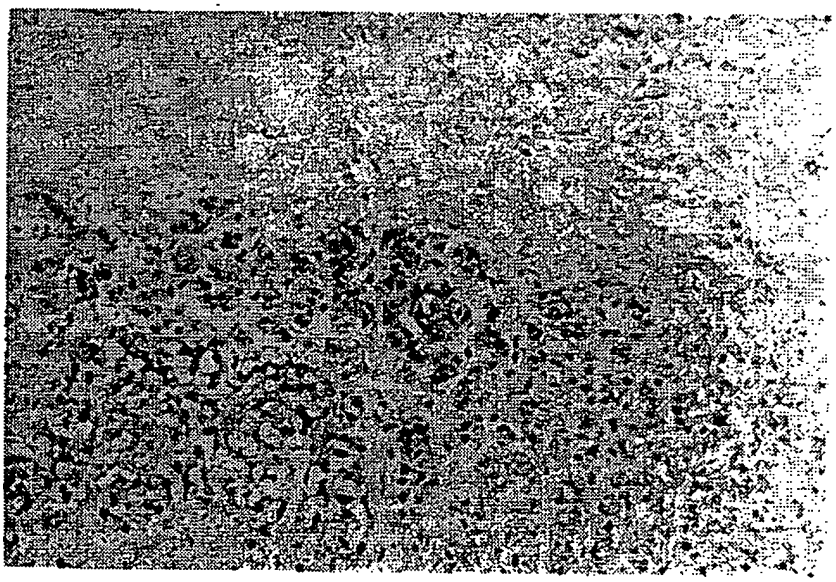


FIG. 3B

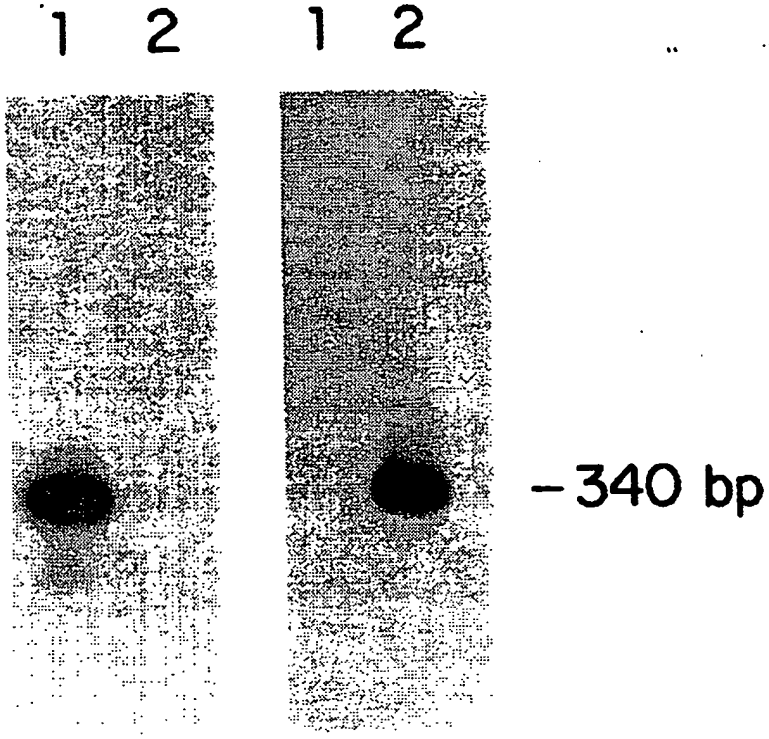


FIG. 4A FIG. 4B

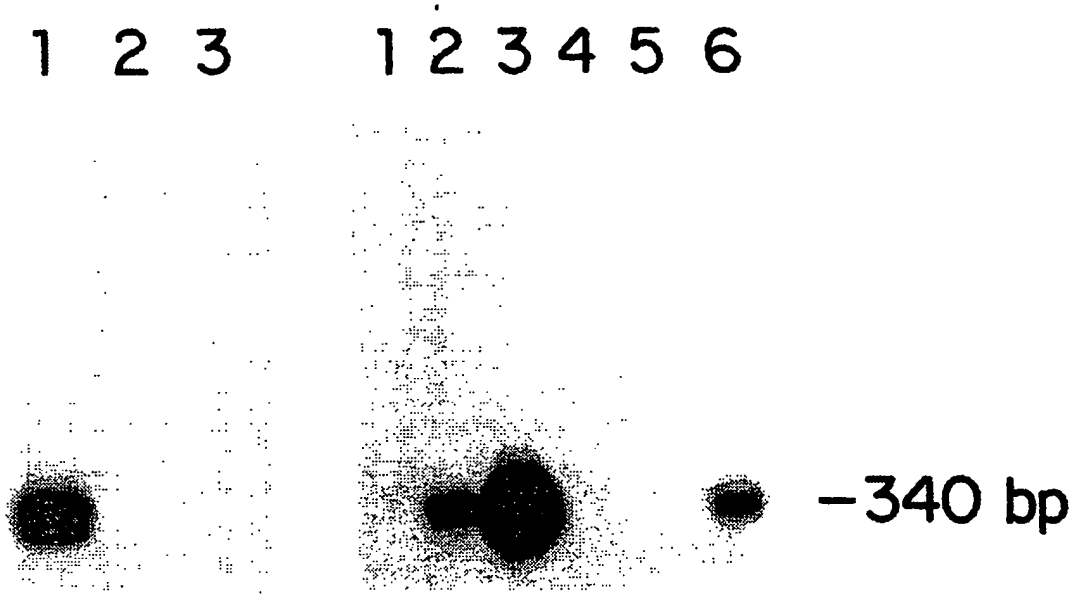


FIG. 5A FIG. 5B

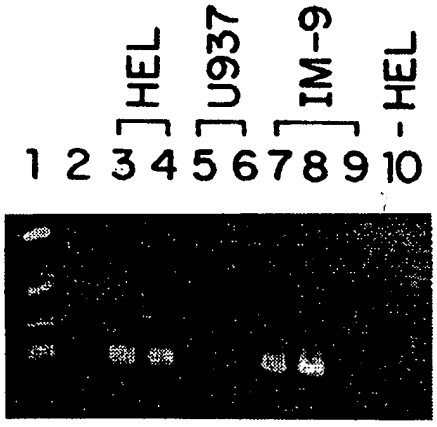


FIG. 6A

FIG. 6B

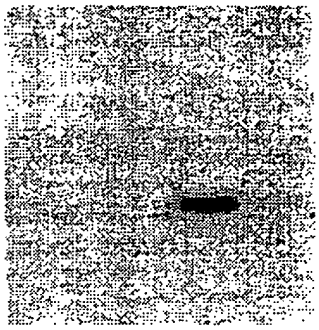
FIG. 6C

FIG. 6D

bp
404
284
340
159

FIG. 7A

5 4 3 2 1



12-LO

FIG. 7B

5 4 3 2 1

GAPDH

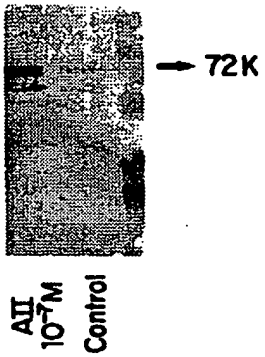


FIG. 8A

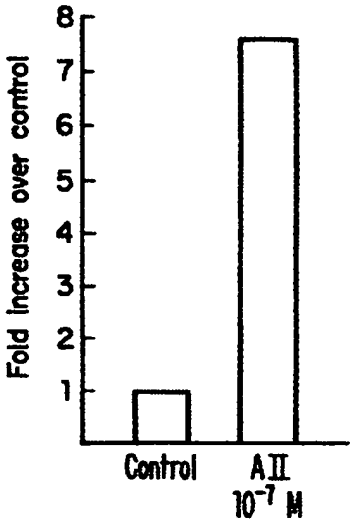


FIG. 8B

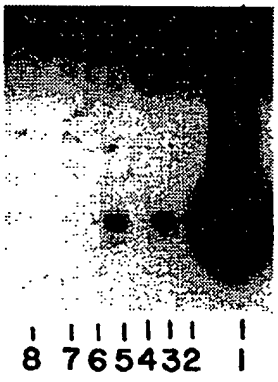


FIG. 9A

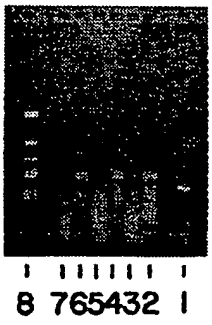


FIG. 9B

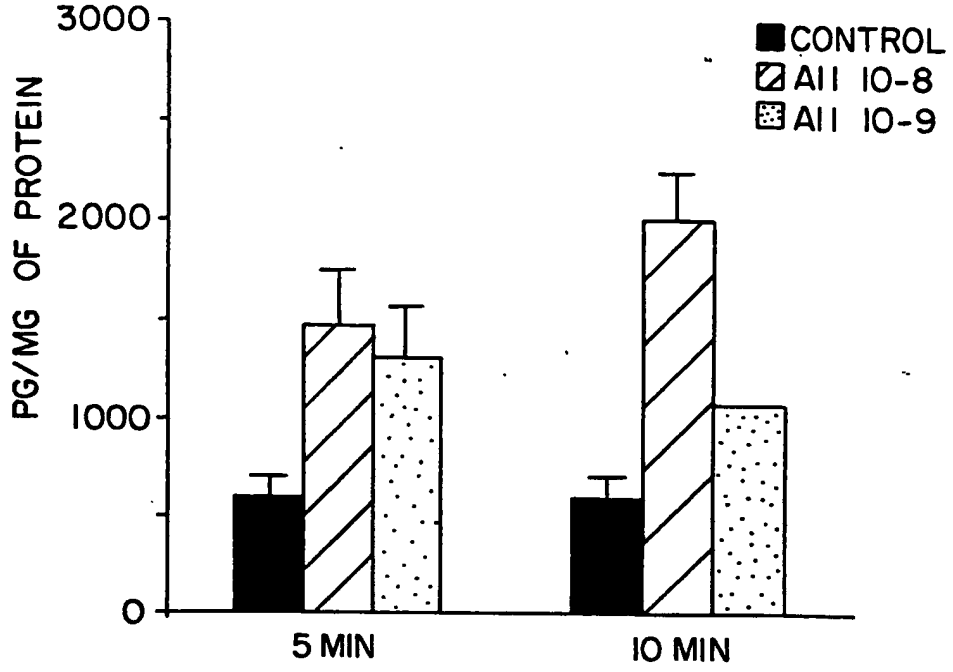

3000
2000
1000
0
PG/MG OF PROTEIN
CONTROL
AII 10-8
AII 10-9
5 MIN
10 MIN

FIG. 10

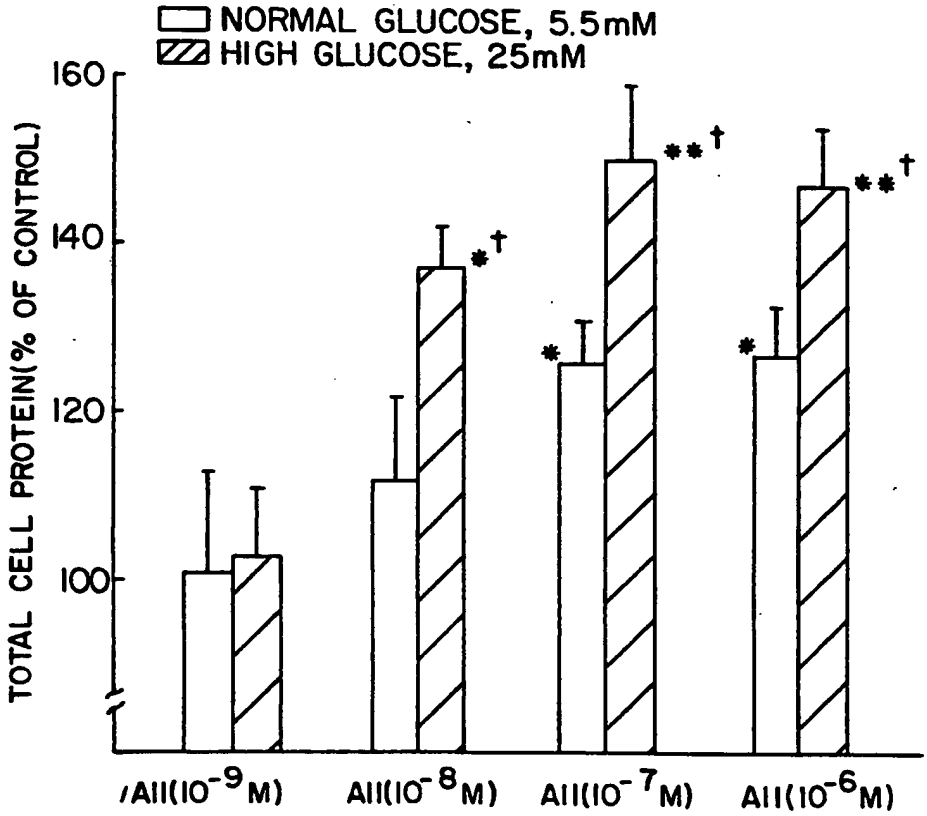

NORMAL GLUCOSE, 5.5mM
HIGH GLUCOSE, 25mM
160
140
120
100
TOTAL CELL PROTEIN(% OF CONTROL)
/AII(10-9M)
AII(10-8M)
AII(10-7M)
AII(10-6M)

FIG. 21

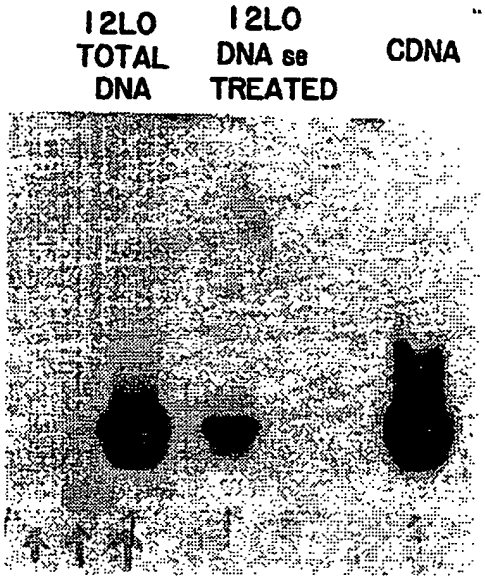


FIG. 11

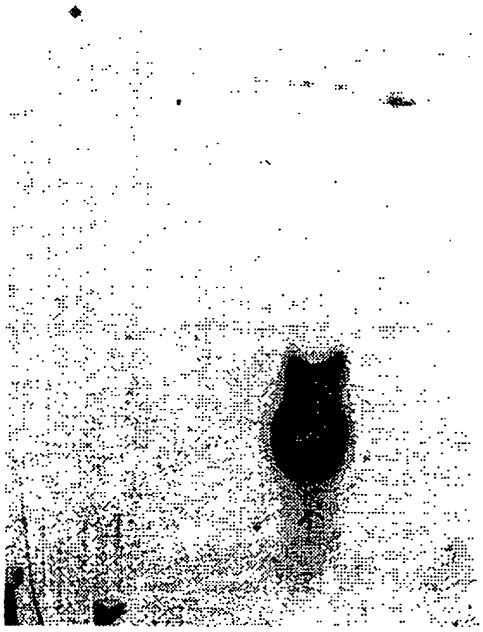


FIG. 12

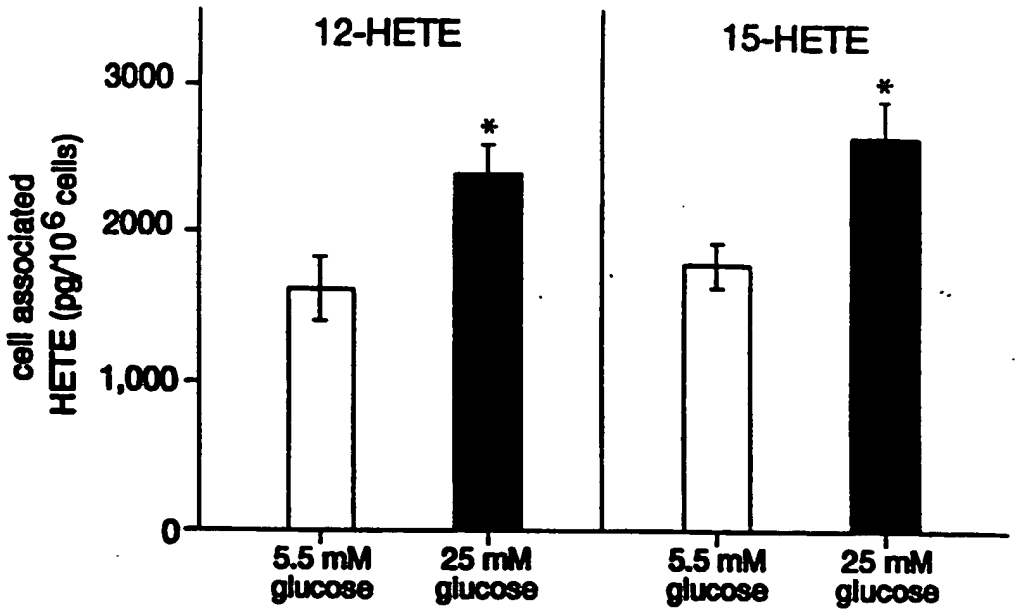

12-HETE
15-HETE
cell associated HETE (pg/10^6 cells)
3000
2000
1,000
0
*
*
5.5 mM glucose
25 mM glucose
5.5 mM glucose
25 mM glucose

FIG. 13

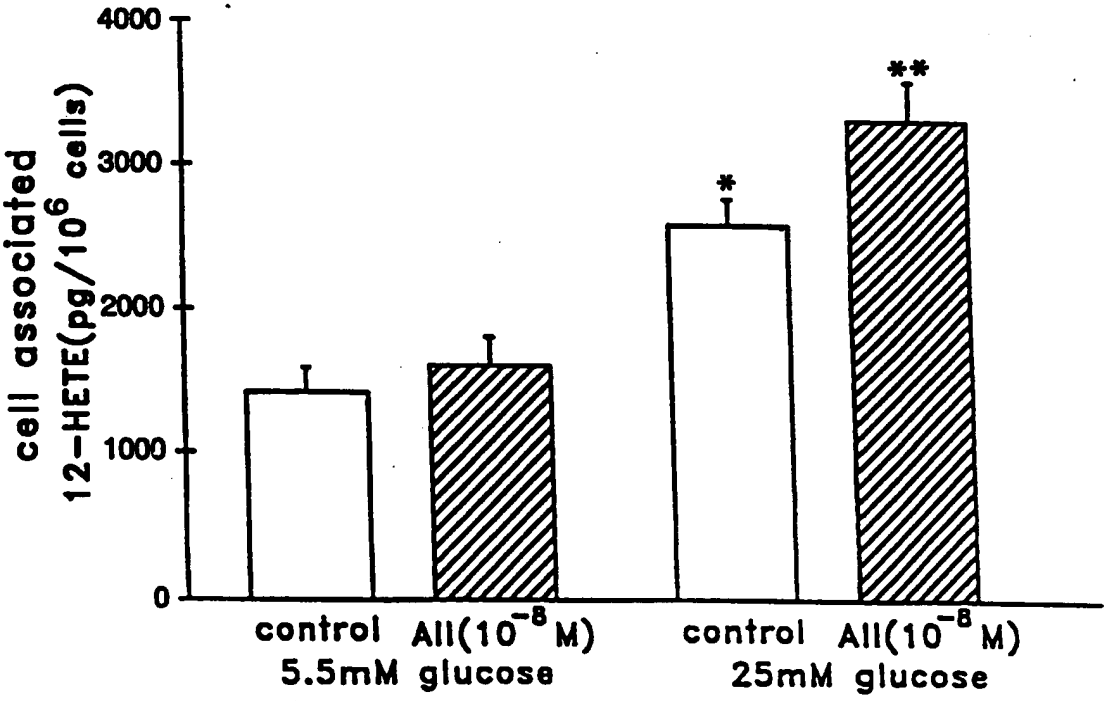

cell associated 12-HETE(pg/10^6 cells)
4000
3000
2000
1000
0
*
**
control
AII(10^-8 M)
5.5mM glucose
control
AII(10^-8 M)
25mM glucose

FIG. 14

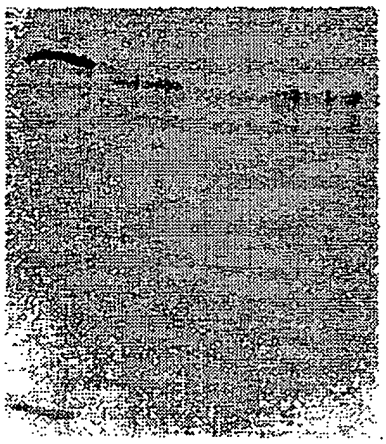


FIG. 15A

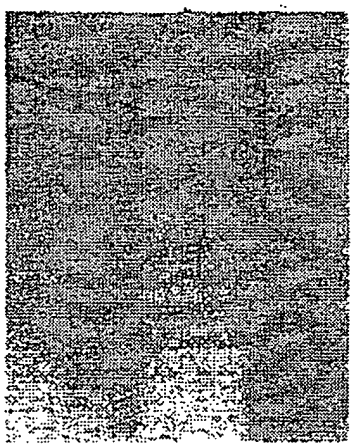


FIG. 15B

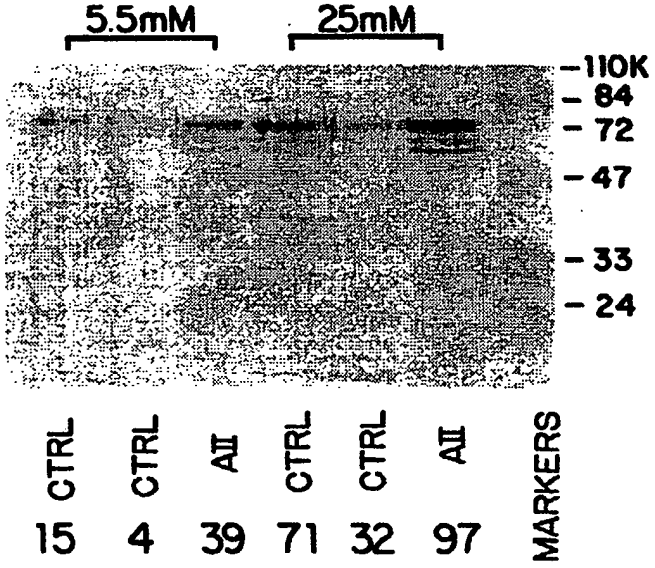


FIG. 16

FIG. 17A

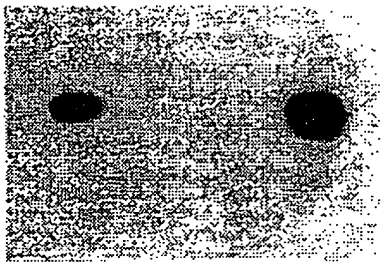


FIG. 17B

FIG. 18A

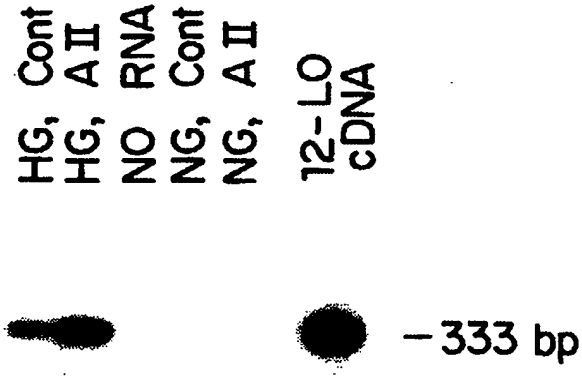


FIG. 18B

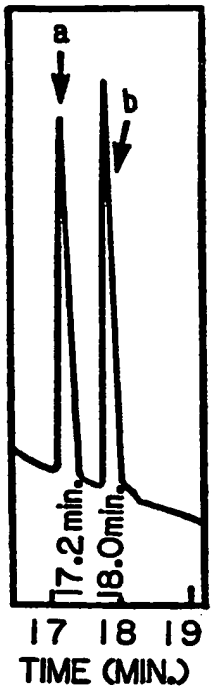


FIG. 19A

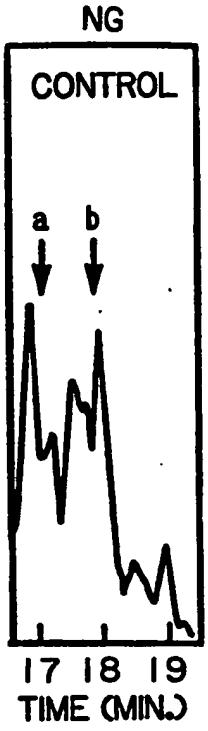


FIG. 19C

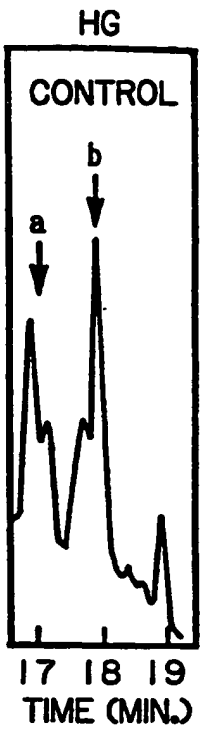


FIG. 19E

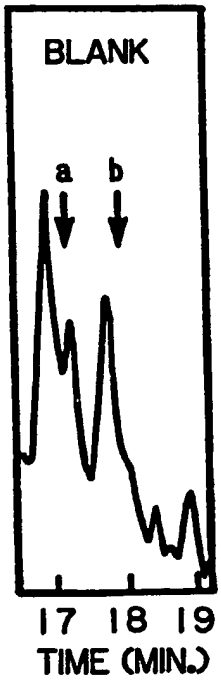


FIG. 19B

FIG. 19D

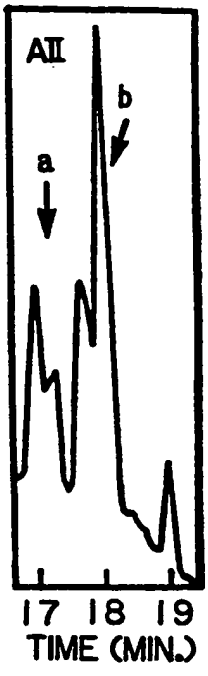


FIG. 19F

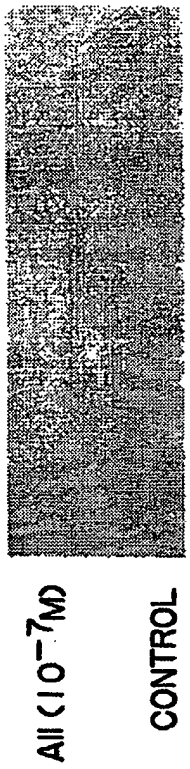


FIG. 20A

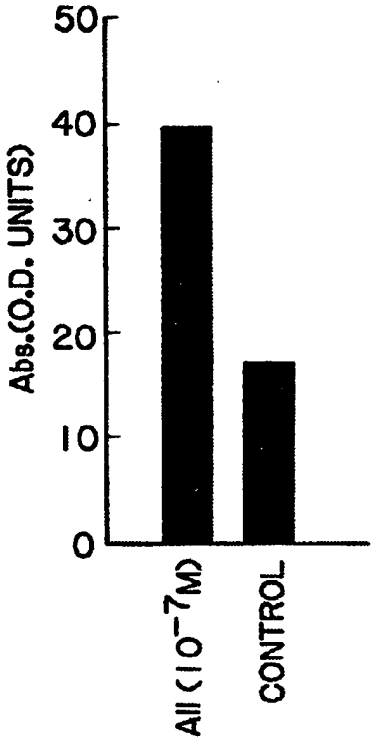


FIG. 20B

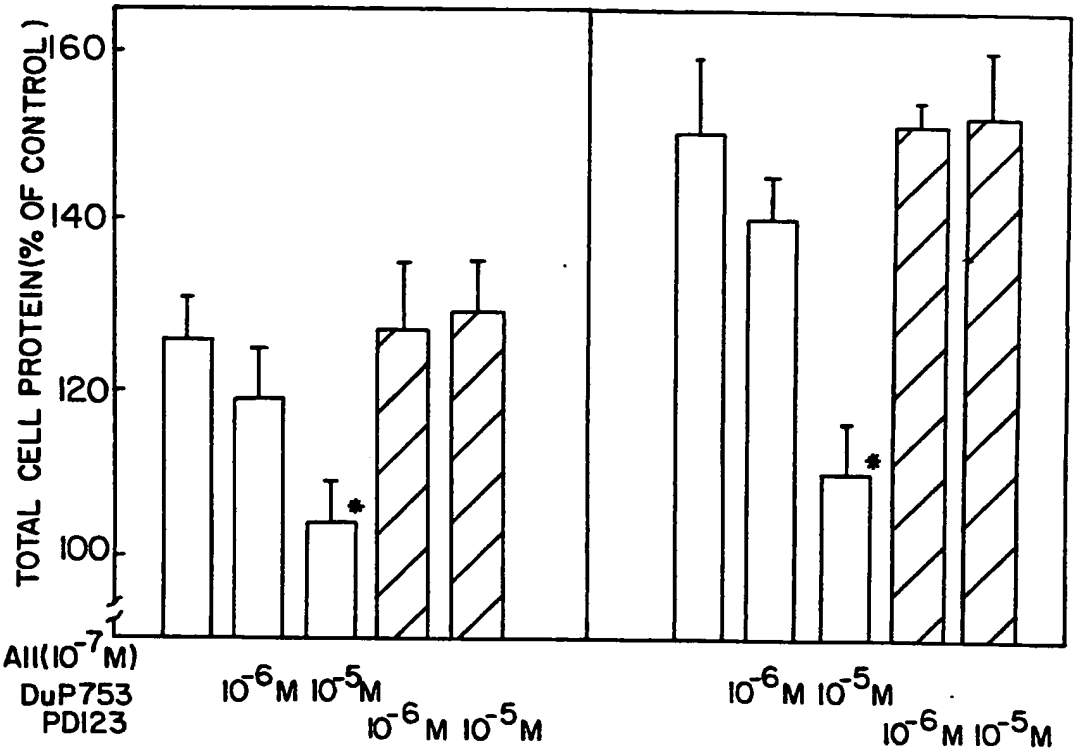

TOTAL CELL PROTEIN(% OF CONTROL)
160
140
120
100
AII(10^{-7}M)
DuP753
PD123
10^{-6}M 10^{-5}M
10^{-6}M 10^{-5}M
10^{-6}M 10^{-5}M
10^{-6}M 10^{-5}M

FIG. 22

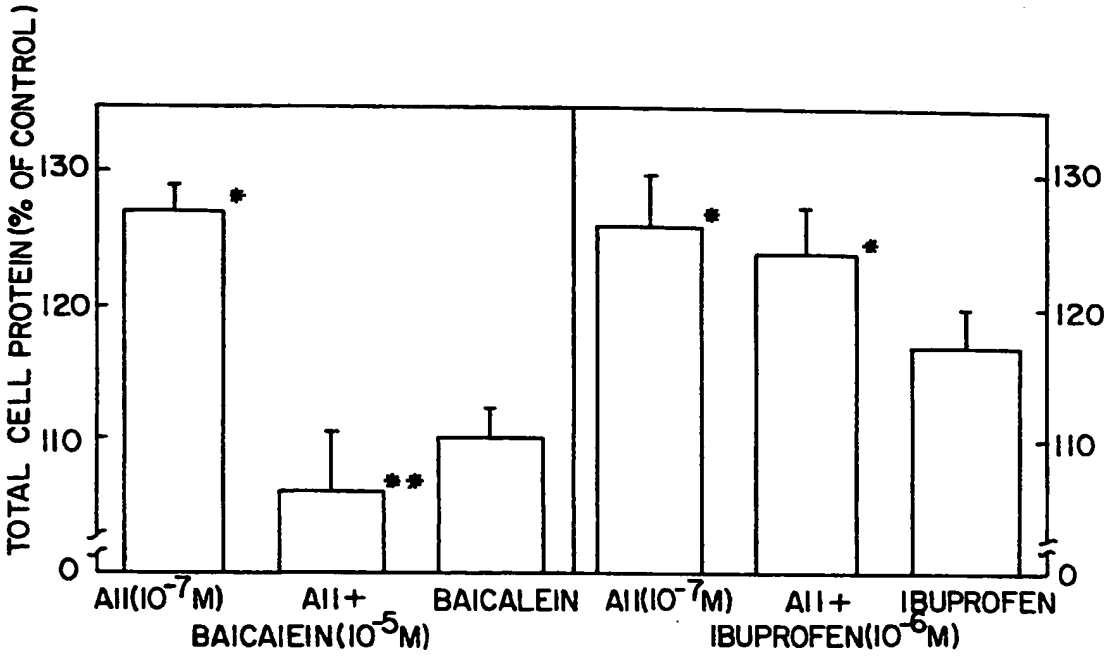

TOTAL CELL PROTEIN(% OF CONTROL)
130
120
110
0
AII(10^{-7}M)
AII+ BAICAIEIN(10^{-5}M)
BAICALEIN
AII(10^{-7}M)
AII+ IBUPROFEN(10^{-6}M)
IBUPROFEN

FIG. 23

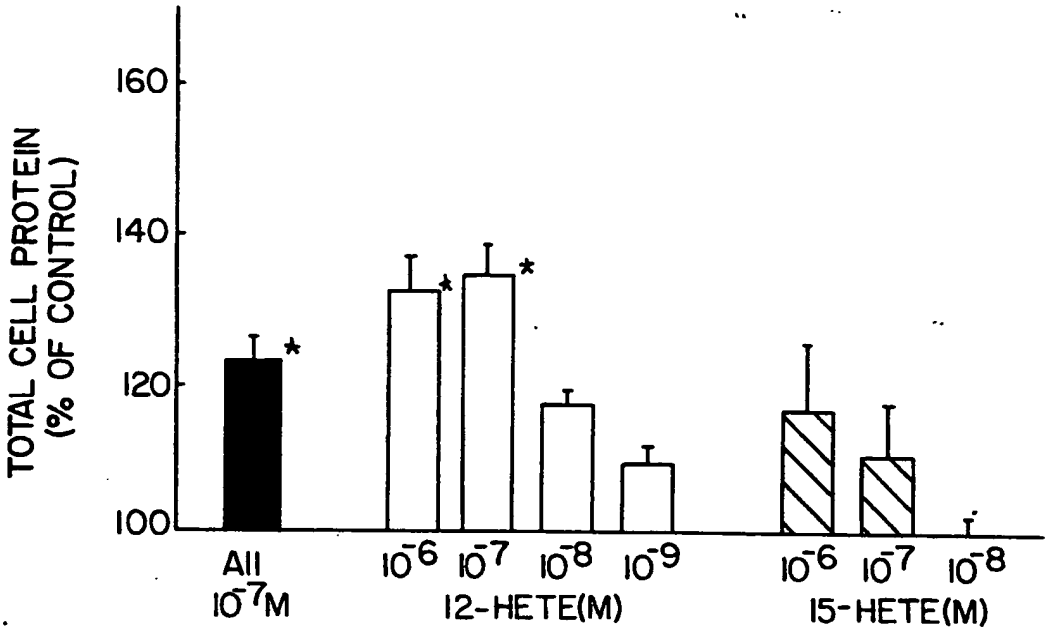


FIG. 24A

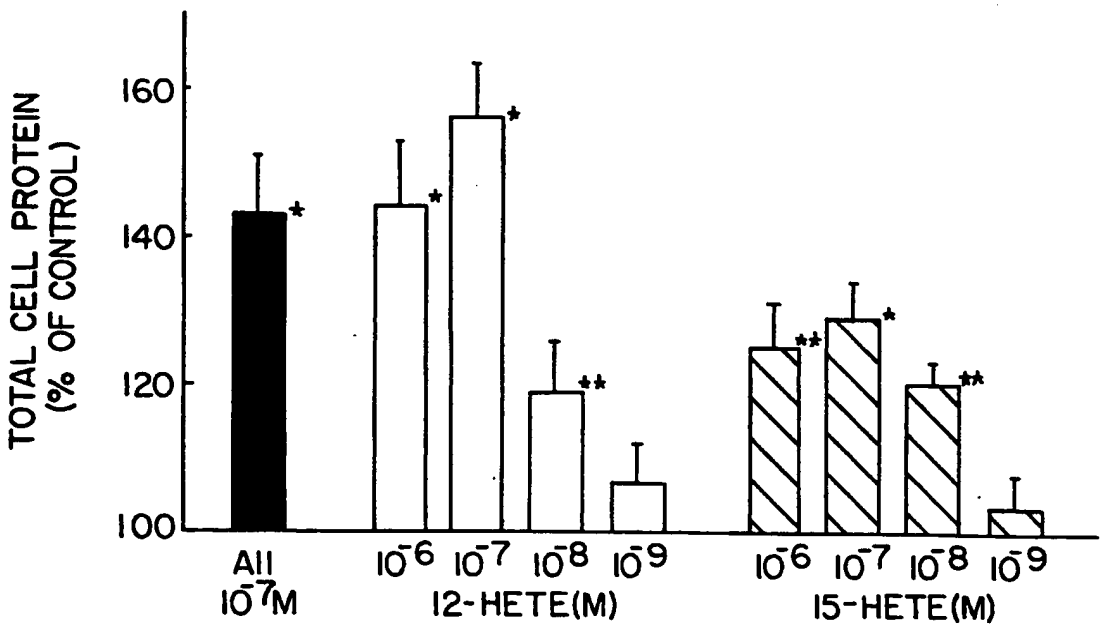


FIG. 24B

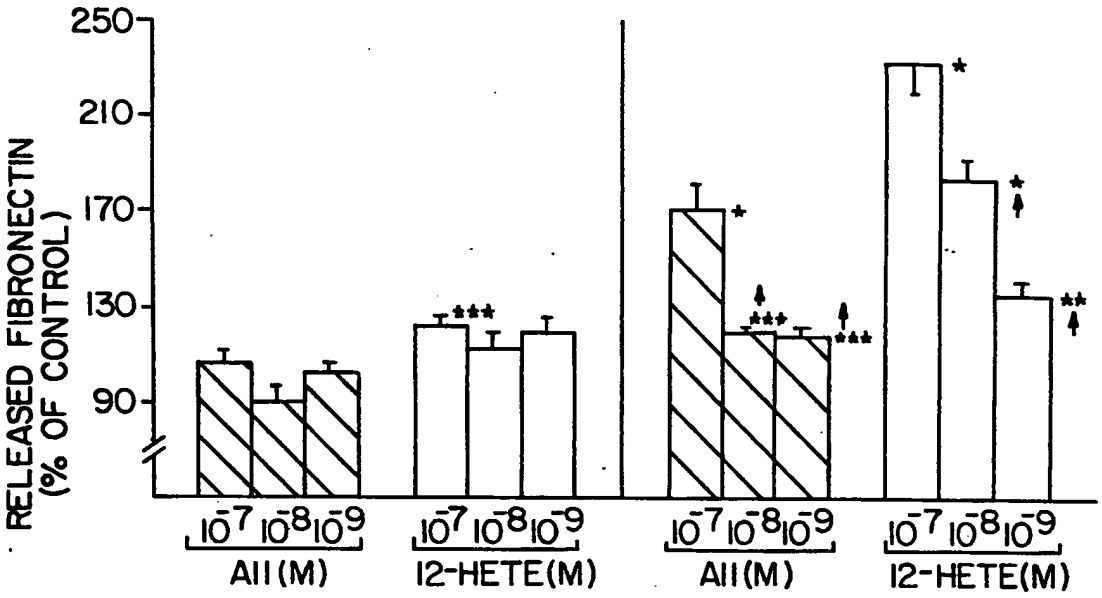


FIG. 25A

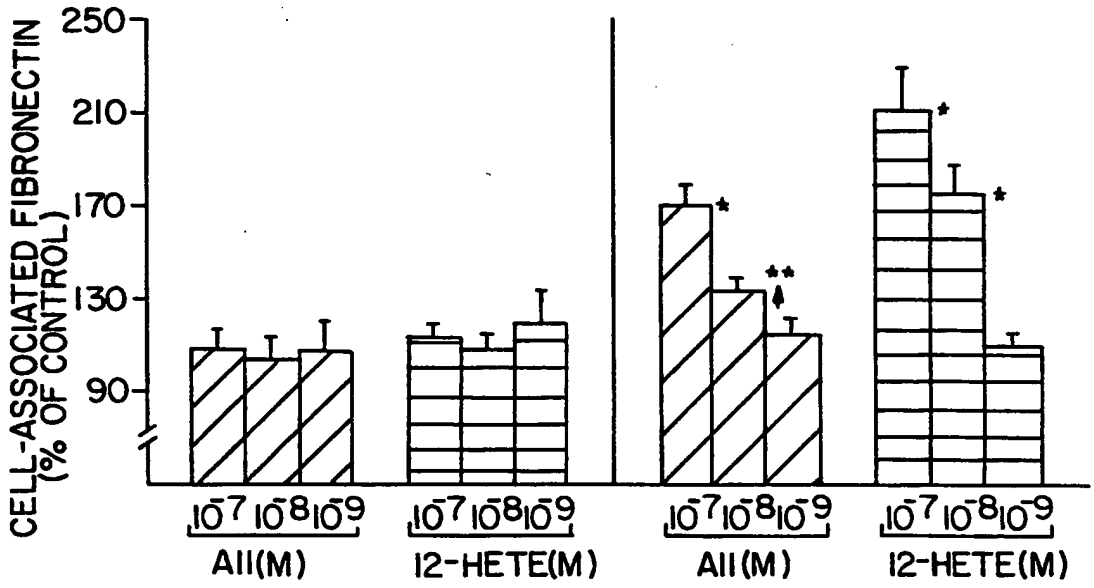


FIG. 25B

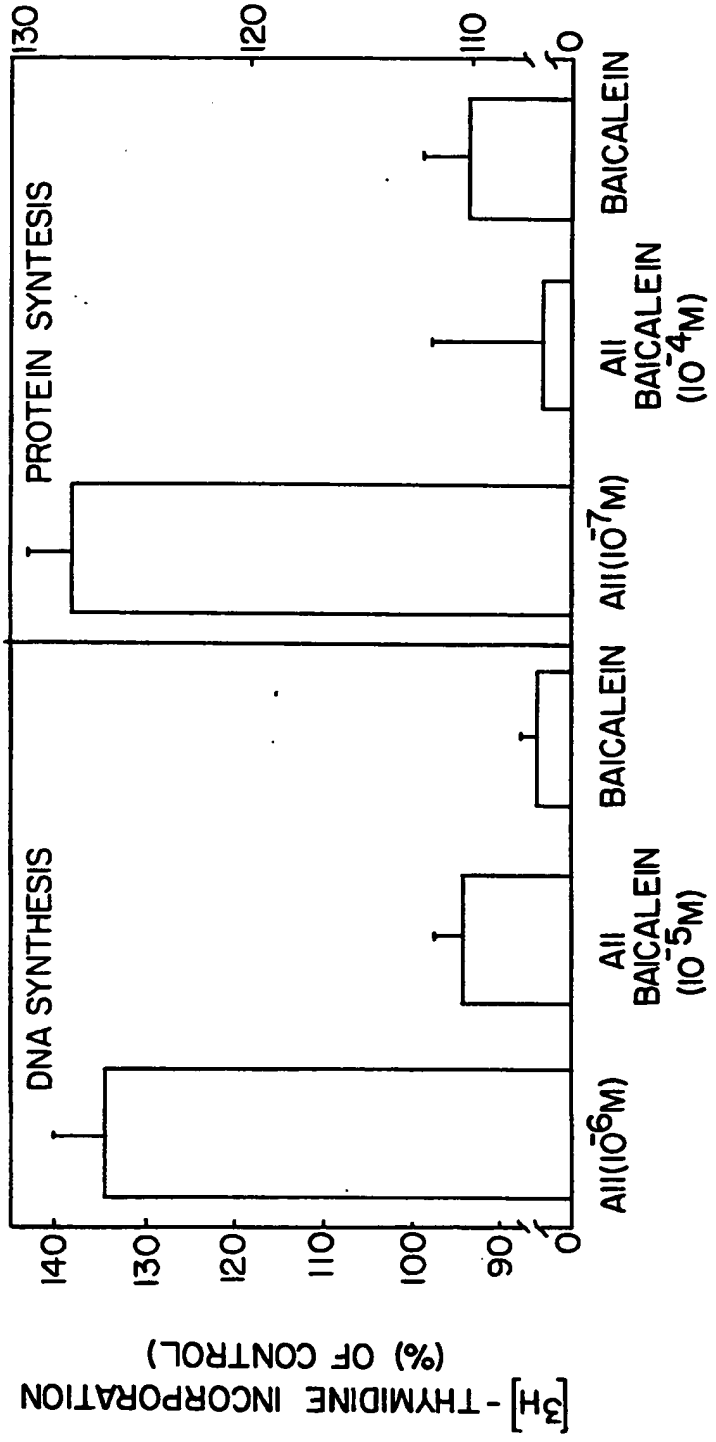

[3H] - THYMIDINE INCORPORATION
(%) OF CONTROL)
140
130
120
110
100
90
0
DNA SYNTHESIS
AII(10-6M)
All BAICALEIN (10-5M)
BAICALEIN
PROTEIN SYNTESIS
AII(10-7M)
All BAICALEIN (10-4M)
BAICALEIN
130
120
110
10

FIG. 26

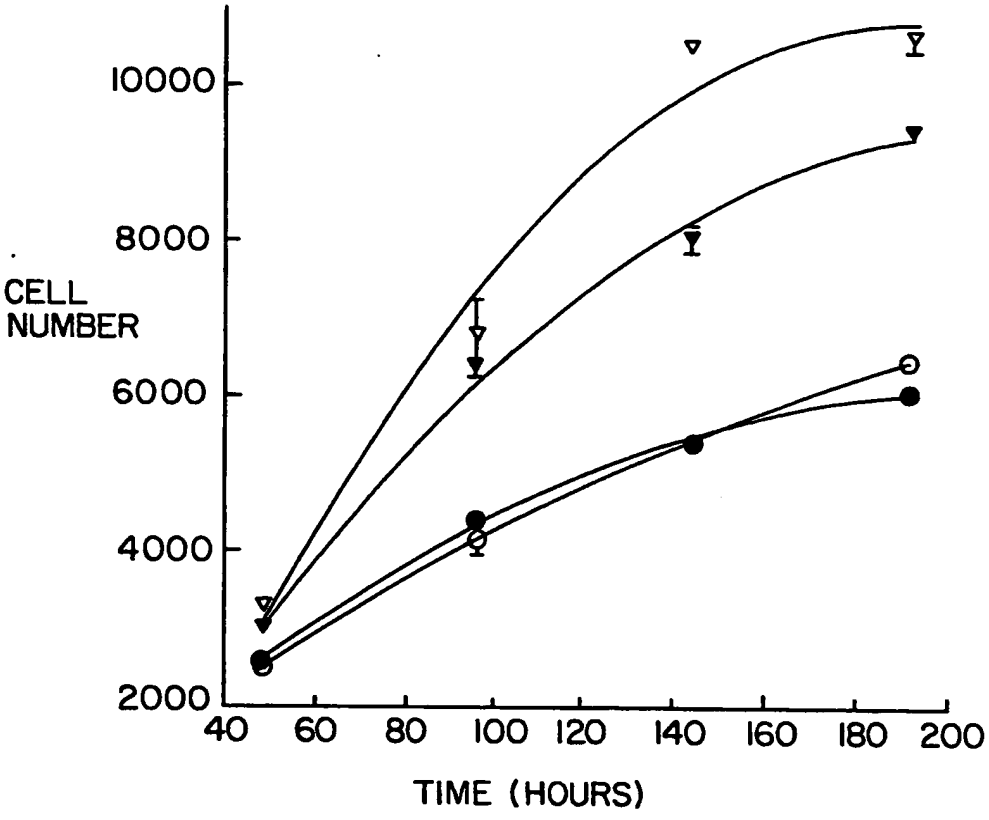

10000
8000
CELL
NUMBER
6000
4000
2000
40
60
80
100
120
140
160
180
200
TIME (HOURS)

FIG. 27

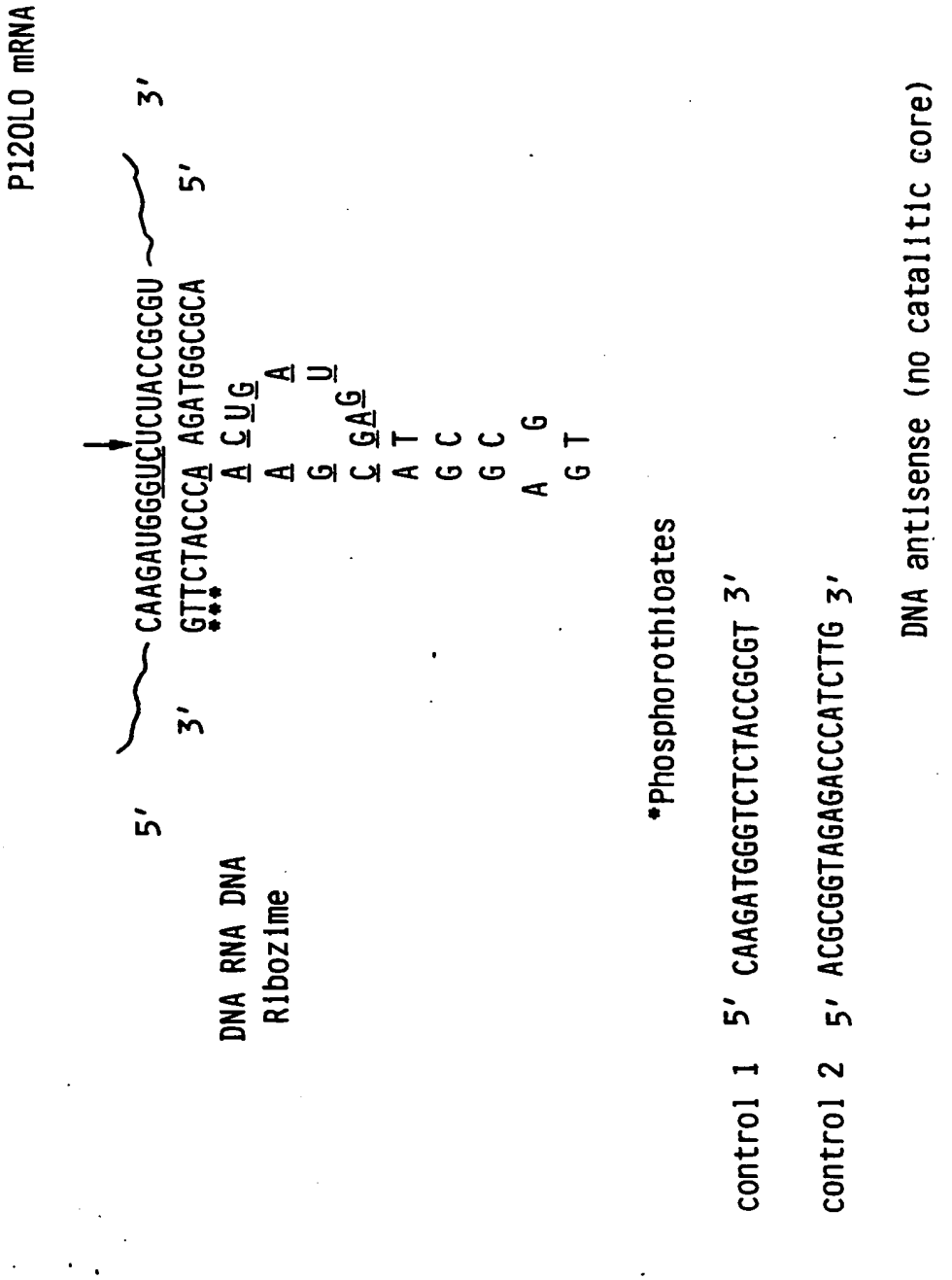

P120LO mRNA
5' CAAGAUGGGUCUCUACCGCGU 3'
3' GTTCTACCCA AGATGGCGCA 5'
DNA RNA DNA
Ribozime
A C U G
A A
G U
C GA G
A T
G C
G C
A G
G T
*Phosphorothioates
control 1 5' CAAGATGGGTCTCTACCGCGT 3'
control 2 5' ACGCGGTAGAGACCCATCTTG 3'
DNA antisense (no catalitic core)

FIG. 28

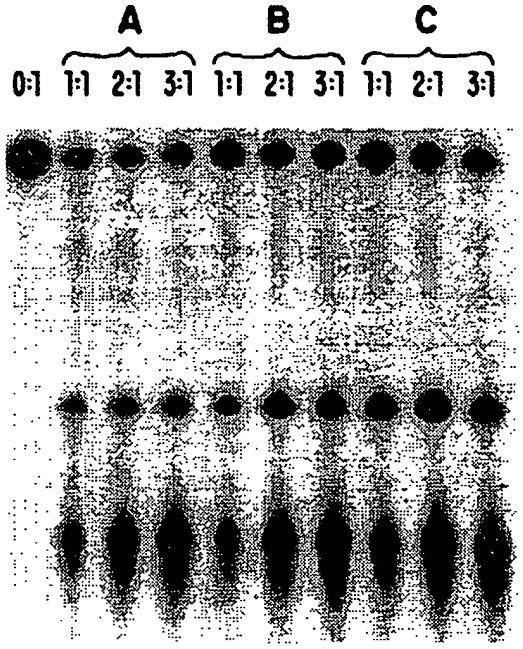

A
B
C
0:1 1:1 2:1 3:1 1:1 2:1 3:1 1:1 2:1 3:1
Ratio of 12-Lo Ribozyme/RNA
32p-5'-12-Lo RNA
Pruduct
12-Lo Ribozyme

FIG. 29

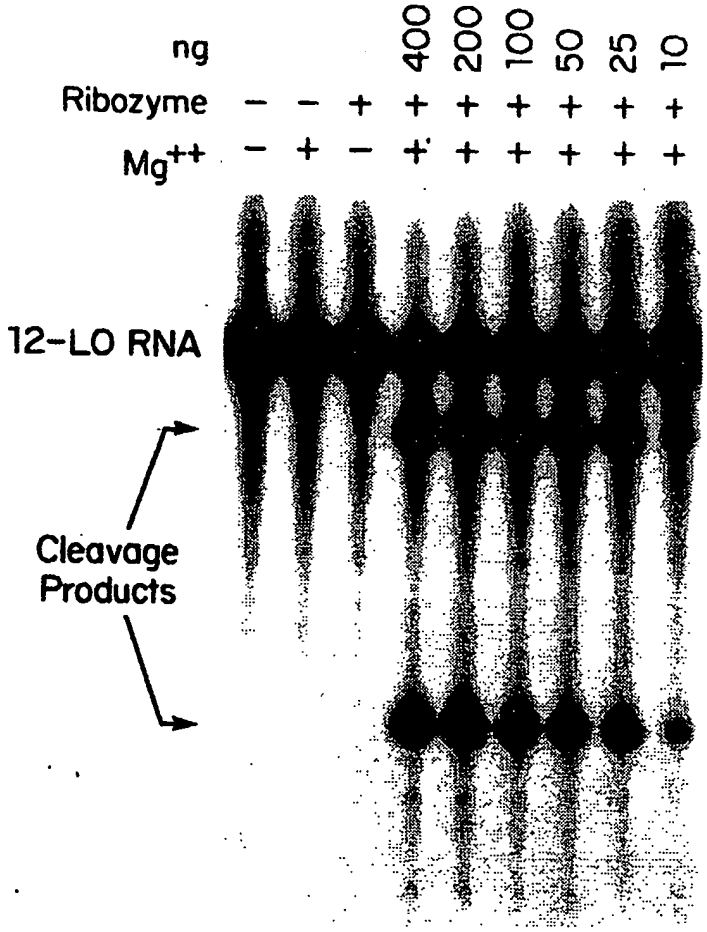

ng
400 200 100 50 25 10
Ribozyme - - + + + + + + +
Mg++ - + - + + + + + +
12-LO RNA
Cleavage Products

FIG. 30

FIG. 31

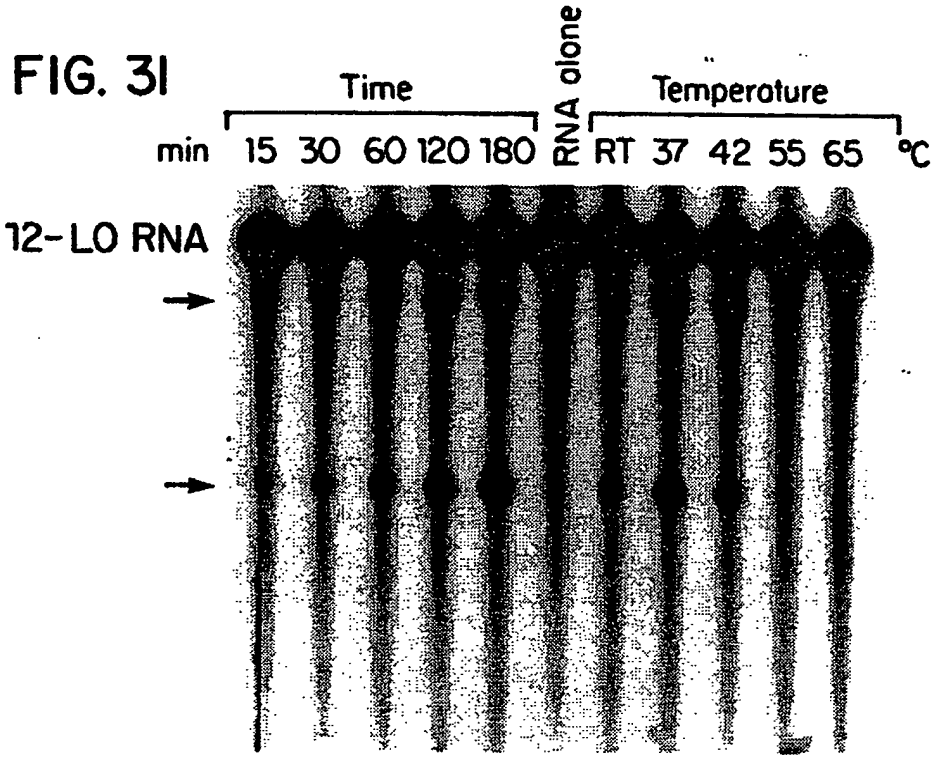
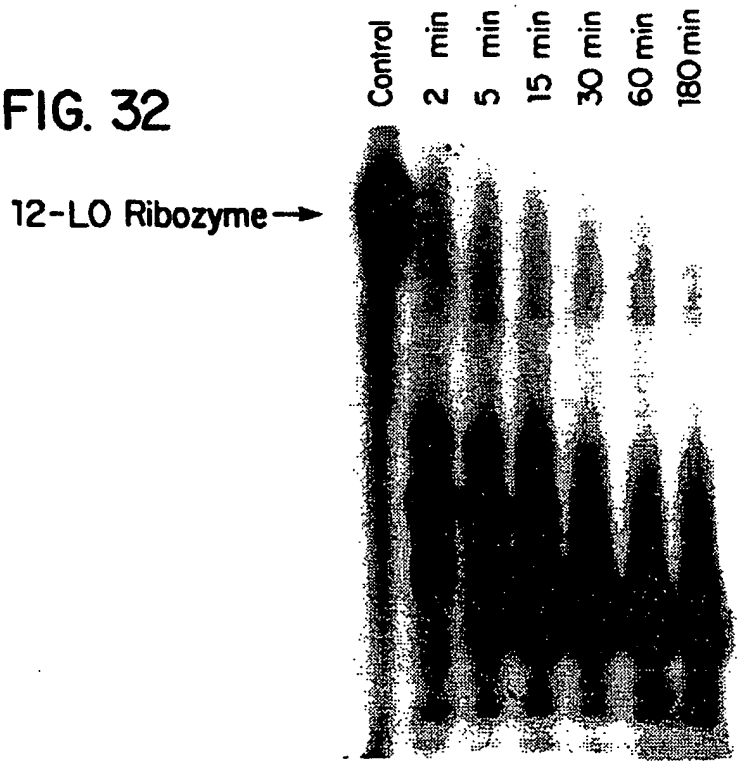

Time
RNA alone
Temperature
min
15 30 60 120 180
RT 37 42 55 65 °C
12-LO RNA

FIG. 32


Control
2 min
5 min
15 min
30 min
60 min
180 min
12-LO Ribozyme

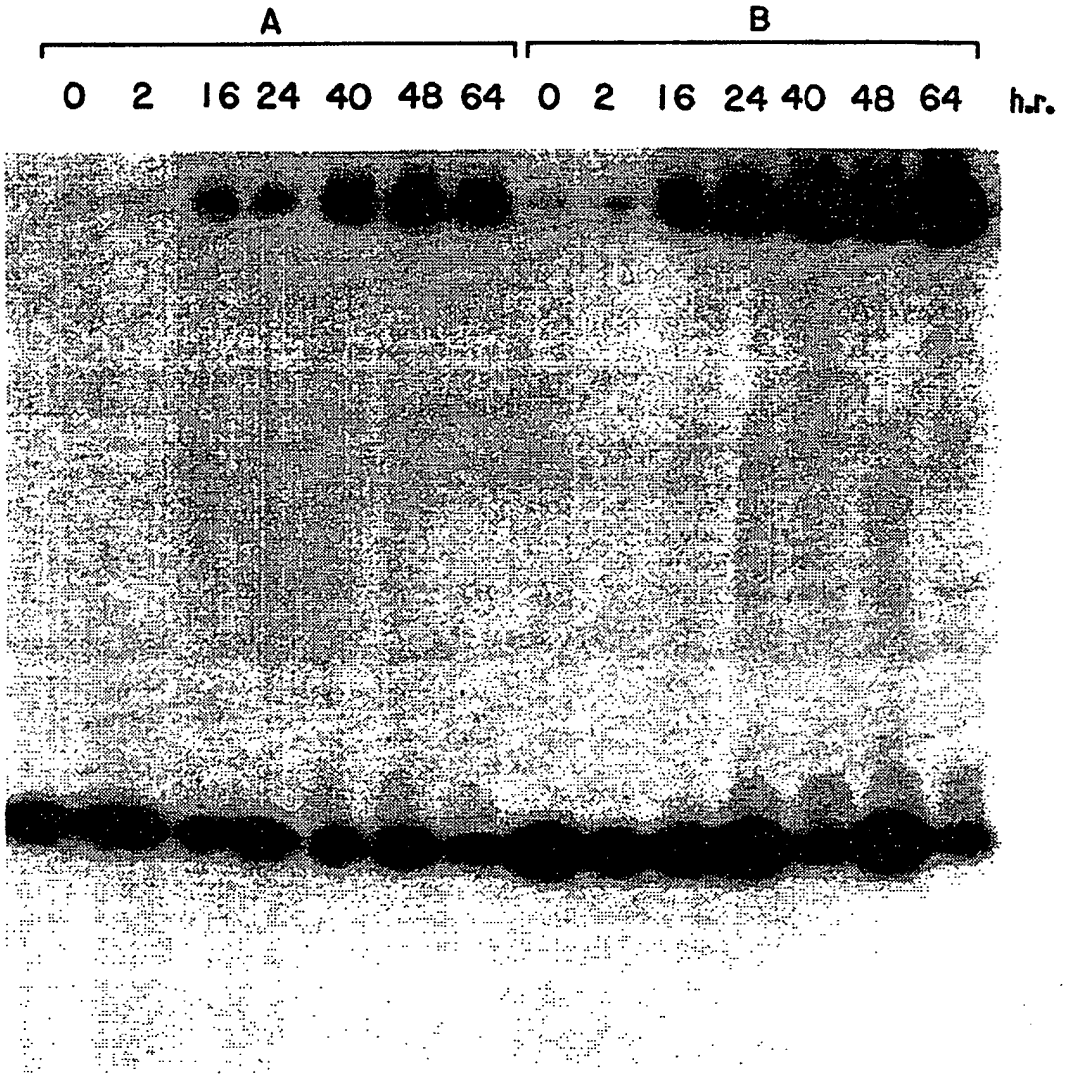


FIG. 33

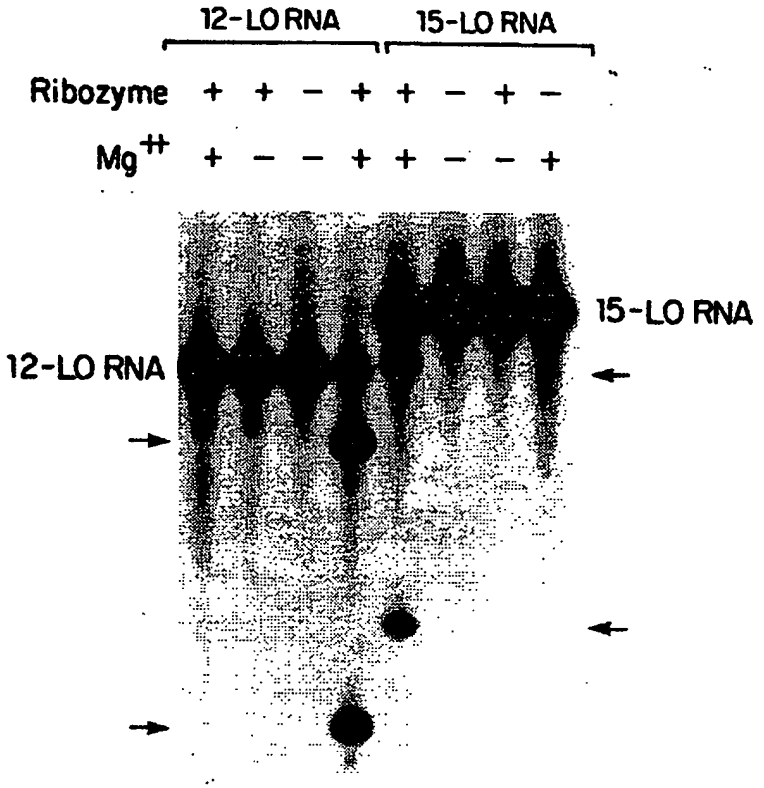

12-LO RNA
15-LO RNA
Ribozyme + + − + + − + −
Mg++ + − − + + − − +
15-LO RNA
12-LO RNA

FIG. 34

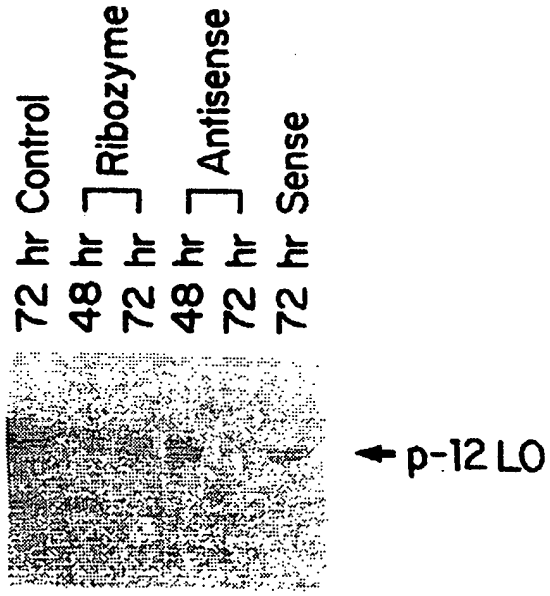

Control
Ribozyme
Antisense
Sense
72 hr
48 hr
72 hr
48 hr
72 hr
72 hr
p-12 LO

FIG. 35

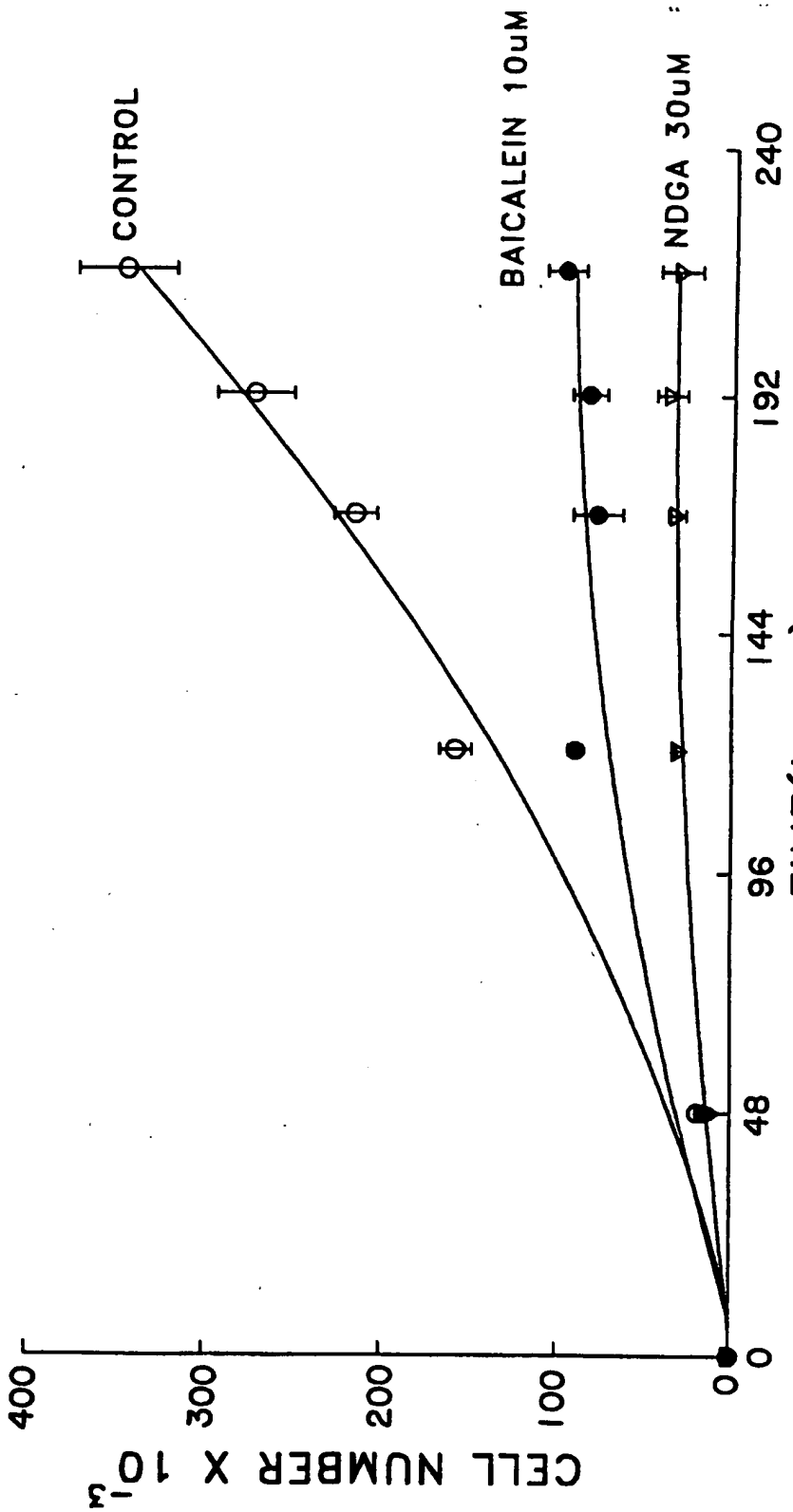

CONTROL
BAICALEIN 10uM
NDGA 30uM
CELL NUMBER X 10^{-3}
400
300
200
100
0
0
48
96
144
192
240
TIME(hours)

FIG. 36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00089

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.6; 435/7.24, 7.4, 25, 189, 240.2; 436/506; 514/3, 342, 456, 632; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, USA, Vol. 87, issued March 1990, T. YOSHIMOTO ET AL, "Cloning and sequence analysis of the cDNA for arachdonate 12-lipoxygenase of porcine leukocytes", pages 2142-2146. See abstract.	10,13
Y	FASEB Journal, Vol. 6, issued 1992, J. GU ET AL, "Evidence for expression of a new form of 12-lipooxygenase (12-LO) in human cells", page A1564, abstract no. 3638. See entire abstract.	10,13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 SEPTEMBER 1994	Date of mailing of the international search report 05 OCT 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer David Saunders, Primary Examiner Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00089

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Proceedings of the National Academy of Sciences, Vol. 90, issued June 1993, R. NATARANJAN ET AL, Elevated glucose and angiotensin II increase 12-lipoxygenase activity and expression in porcine aortic smooth muscle cells", pages 4947-4951. See abstract and page 4951, cols 1-2.	11-12, 14-17
X	Endocrinology, Vol. 134, No. 1, issued January 1994, J. GU ET AL, "Evidence that a leukocyte type of 12-lipoxygenase is expressed and regulated by angiotensin II in human adrenal glomerulosa cells", pages 70-77. See abstract.	14-15
X Y	Prostaglandins Leukotrienes and Essential Fatty Acids, Vol. 45, issued 1992, P.-P.H. LEE ET AL, "Regulation of proliferation of rat mammary tumor cells by inhibitors of cyclooxygenase and lipoxygenase", pages 21-31. See abstract.	7 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/00089

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 31/34, 31/44, 31/155, 37/26, 37/54; C07H 17/00; C12N 5/00, 9/02; C12Q 1/26; G01N 33/564

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/94.6; 435/7.24, 7.4, 25, 189, 240.2; 436/506; 514/3, 342, 456, 632; 536/23.2

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

BIOSIS
12(W)lipoxygenase# and autoimmun? or autoantibod? or diabet?
" and leukocyte# or monocyt? or mononuclear or smooth (w) muscle or adrenal or endotheli?
" and pdgf or platelet(w)derived(w)growth(w)factor
APS 12(w)lipoxygenase

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-6 and 10, drawn to immunoassays and an isolated lipoxygenase used as an antigenic composition therein, classified in Class 435, subclass 7.4.

II. Claims 7-8 and 11-12, drawn to body treatments administering a lipoxygenase inhibitor (e.g. a ribozyme), classified in Class 424, subclass 94.6.

III. Claim 9, drawn to the administration of PDGF and monitoring via a lipoxygenase assay, classified in Class 435, subclass 25.

IV. Claim 13, drawn to RNA encoding lipoxygenase, classified in Class 536, subclass 23.2.

V. Claims 14-15 drawn to a cell culturing method, classified in Class 435, subclass 240.2.

VI. Claims 16-17 drawn to body treatments via glucose concentration regulation, classified in Class 514, subclass 3.

The immunoassays of Group I involve no steps in common with the body treatment steps of Groups II, III and VI or with the culturing method of Group V. The immunoassays of Group I do not employ the RNA composition of Group IV. The body treatments of Groups II, III and VI employ differing agents and achieve differing goals; these treatments do not use the compositions of Groups I or IV. Accordingly the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☑ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.